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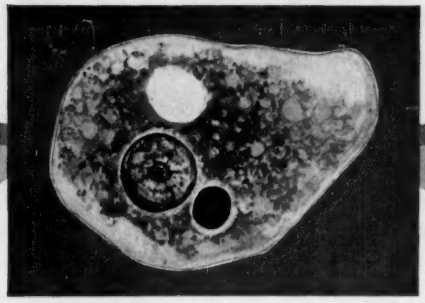

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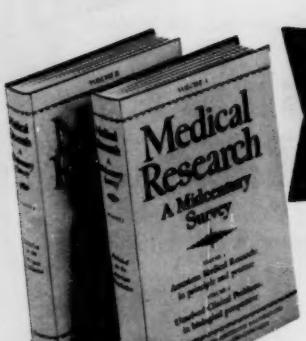

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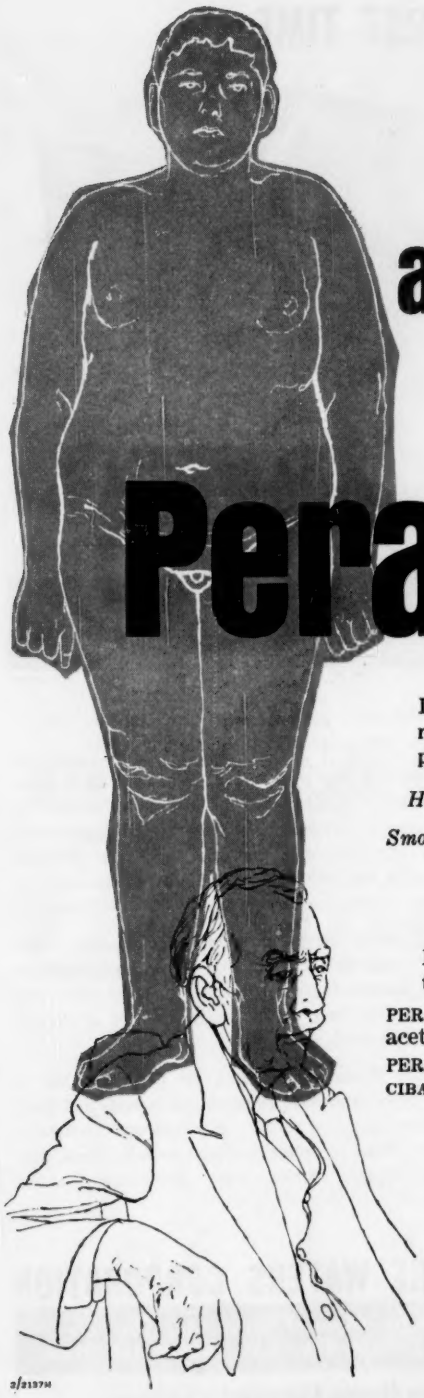

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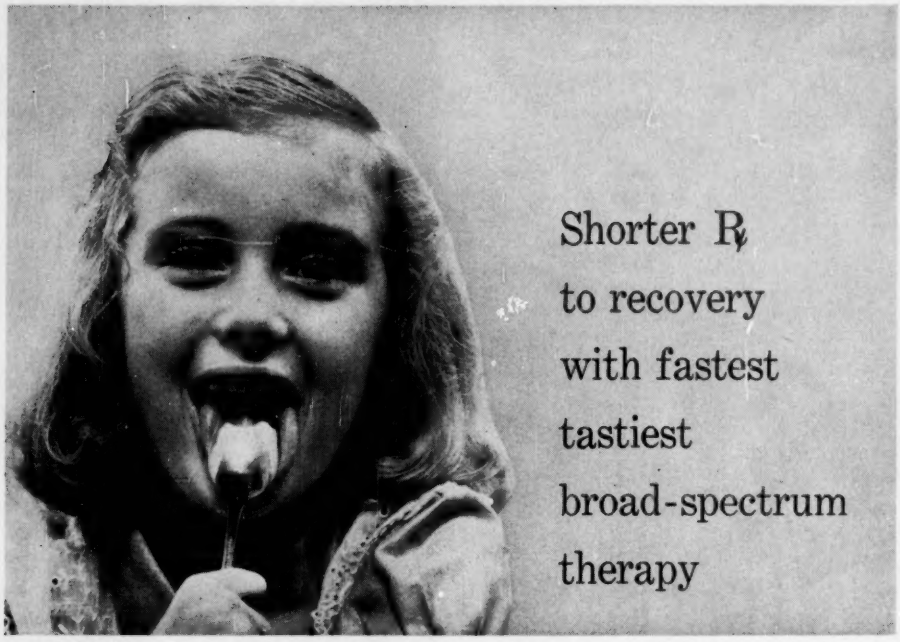

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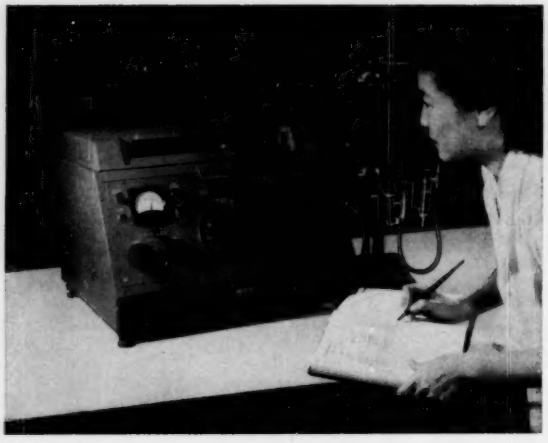

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FATIGUE OF THE SWEAT GLANDS

By JØRN HESS THAYSEN and IRVING L. SCHWARTZ

(From the Hospital of The Rockefeller Institute for Medical Research, New York, N. Y.)

(Submitted for publication July 14, 1955; accepted July 28, 1955)

The rate of sweat secretion has been shown to decrease during prolonged exposure to heat (1–3). This phenomenon has practical importance in the etiology of heat stroke, since the evaporation of sweat is the only significant means for dissipating heat when the environmental temperature equals or exceeds the body temperature.

It is not known whether this decrease in sweating rate is due to a failure of the sudomotor center or to an exhaustion of the peripheral glandular units. The present investigation was undertaken in an attempt to answer this question. The results indicate that the decrease in sweating rate is due primarily to fatigue of the secretory mechanism of the glands. Apparently sweating can continue indefinitely at the same rate only as long as the demand upon the secretory mechanism does not exceed the capacity of the simultaneous restorative processes. Whenever the demand upon the secretory mechanism exceeds restorative capacity the rate of sweating decreases.

PROCEDURE

Four young, un-acclimatized, healthy males served as subjects for the experiments which were conducted during the winter months. Sweating was stimulated in a hot room, temperature 40° to 45° C., relative humidity 60 to 90 per cent. The carbon dioxide and oxygen concentrations of the air were controlled and kept constant at normal values prevailing outside the gas-tight chamber. The subjects entered the room nude except for a pair of light shorts and remained at rest lying uncovered on top of a bed during the entire experiment. Room temperature and rectal temperature were recorded every 30 minutes.

The total amount of sweat secreted from the entire body surface was estimated at accurately timed intervals of 30 to 60 minutes' duration. The subject removed his shorts. His skin was dried thoroughly with towels and body weight measured to the nearest 50 gm. Water, approximately at body temperature, was imbibed in amounts equivalent to the loss in body weight; sodium chloride was taken to replace an estimated average loss of 50 mEq. of sodium per liter of sweat produced. The total amount of sweat secreted in any period was calculated from the weight loss with corrections for water intake and urine flow. The insensible water loss from the skin and through the expired air and the metabolic weight loss were taken into account and did not introduce a significant error into this calculation at the sweating rates which prevailed in these experiments.

Local collections of sweat, using special collection chambers (4), were obtained from the ventral surfaces of the forearms. The sweat was absorbed into filter paper discs and the rate of flow calculated by the weight increment of the disc during exactly timed periods of 20 to 30 minutes' duration.

Prints of the sweating areas were obtained and analyzed by methods previously described (5). In brief, paper impregnated with iodine was placed in special holders and pressed against the skin of the local collection areas for timed periods of 1 to 10 seconds. The water emerging from the sweat glands reacted with the iodine and starch of the paper to form distinct blue dots. These were counted under a dissecting microscope (coefficient of variation for replicate counts = 2 per cent), and classified by size with increments in diameter of 0.04 mm. The dispersion of glandular activity was shown by the distribution of the logarithm of the dot diameter, this distribution being chosen because it is independent of the absolute rate of sweating and the length of exposure of the printing paper (5).

The reactivity of the sweat glands to substances with a stimulating or inhibiting effect on sweat secretion was tested by injection of a minute dose at the site of sweat collection on the forearm. The symmetrical site on the other extremity served as a control, since the amount of drug injected at the test site was too small to elicit systemic effects.

RESULTS

The rate of sweating decreased and the rectal temperature rose after 3 to 6.5 hours of profuse sweating.

The decrease in sweat flow after long exposure to heat was observed in four experiments lasting 5 to 6.5 hours. As shown in Figure 1 the total output of sweat rose to a maximum value by the third to fifth hour, and then decreased to 30 to 60 per cent of the maximum despite an increase in temperature of the hot room. Flow rates measured with collecting chambers on the forearms, showed a rise and fall that paralleled the general trend. Air temperature and rectal temperature

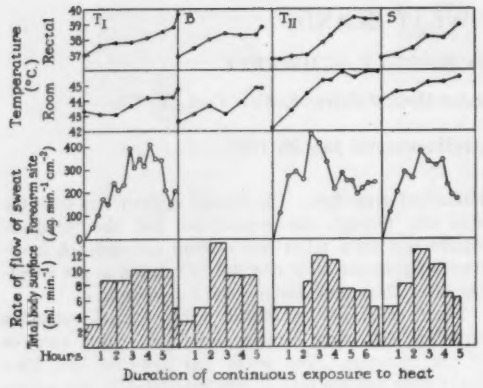

FIG. 1. THE RATES OF SWEATING (TOTAL BODY SURFACE AND LOCAL COLLECTION SITES ON THE FOREARMS), ROOM TEMPERATURES AND RECTAL TEMPERATURES ARE SHOWN IN RELATION TO TIME SPENT IN A HOT ROOM (FOUR EXPERIMENTS, THREE SUBJECTS)

rose throughout the course of each experiment, showing that the decrease in sweating rate was not due to a decrease in primary stimulus.

The decrease in the rate of sweating was mainly due to a decrease in the mean flow per gland and less to a decrease in the number of functioning glands; the relative activities[1] of the sweat glands remained unchanged throughout the heat exposure. This phenomenon was demonstrated in experiment T_{II} by simultaneous collections of sweat and registrations of the number of functioning glands within the same area of skin of the forearm. As shown in Figure 2 and Table I, the initial increase in sweating rate was effected chiefly by an increase in the mean flow per gland and to a lesser extent by increase in the number of functioning glands. The subsequent fall in the rate of sweating was, likewise, more due to decrease in the mean flow per gland and less to decrease in the number of functioning glands (Table I). The relative activities of the glands remained unchanged despite the changes in mean output, indicating that each of the individual units fatigued at the same rate. The relative contributions of small and large glands were similar at all rates of flow (Figure 2).

The output of sweat, following intradermal injection of Mecholyl was diminished by preliminary prolonged reflex sweating. In one of the above experiments (T_I) Mecholyl (β-methylacetylcholine hydrochloride), 2 mg. in 0.5 ml. isotonic saline, was injected intradermally at the site of local sweat collection on the forearm after a period of six hours of profuse reflex sweating. The output of sweat was compared to the average output at the same site when stimulated with Mecholyl under "resting conditions."

It will be seen from Figure 3 that the reactivity of the sweat glands to this standard dose of cholinergic drug was significantly decreased by preliminary prolonged reflex sweating. Repetition of this experiment yielded identical results.

Intradermal infiltration with atropine, sufficient to block sweat secretion for a period of 4.3 hours, was followed by hypersecretion of sweat as compared to a symmetrical site that had continued to function throughout a prolonged exposure. Sweat collection chambers were glued to symmetrical

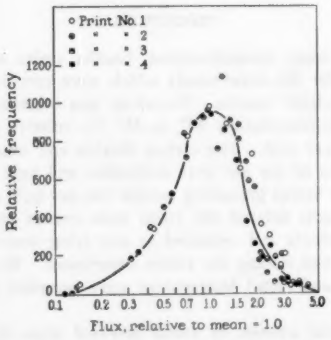

FIG. 2. THE FREQUENCY DISTRIBUTION OF THE LOGARITHM OF DOT DIAMETERS OF FOUR PRINTS TAKEN FROM THE SAME FOREARM SITE AT VARIOUS TIMES DURING EXPOSURE TO HEAT

On each print the number of glands was counted and classified by increments in diameter of 0.04 mm. The logarithms of the dot diameters were used and the distributions of the four prints shifted to a common mean so that each distribution is independent of the sweating rate and the printing time.

[1] The term relative activity denotes the fraction of the total flow from an area contributed by each of the individual glands. Therefore it refers to the secretory activity of any gland relative to the secretory activity of every other gland as well as the field as a whole. For example a small gland in a field of 100 glands may contribute 0.1 per cent of the total outflow, an average gland 1 per cent, and a large gland 10 per cent; these relative contributions may remain the same or change with varying physiologic or pathologic conditions.

sites on the ventral surfaces of the forearms. Atropine sulfate (0.005 mg. in 0.5 ml. isotonic saline) was injected intradermally at the collection site on the left arm. Thereafter the subject entered the hot room where he remained for 6.8 hours. The blocking effect of the atropine was checked by making prints with iodinated paper. Whenever the faintest trace of sweating was observed, atropine was reinjected (usually every 30 to 40 minutes). These injections had no systemic effect as judged by the overall sweat secretion and the rate of sweating from the symmetrical site on the right forearm. The atropine injections were repeated eight times, blocking the secretion of sweat at the collection site on the left forearm for a total of 4.8 hours. Then the atropinized area was permitted to sweat freely for the remaining 2 hours of the stay in the hot room. Sweat was collected in 30-minute periods from the collection site on the right arm starting 15 minutes after the subject entered the hot room and ending 6.5 hours later. For the last 2 hours sweat collections were made in 20-minute periods from both forearm sites.

TABLE I

Relation of the number of functioning glands to the mean sweating rate from an area of forearm skin 1 inch in diameter at various times during exposure to heat

Print number	Time after entering hot room *hrs.*	Gland count	Mean sweating rate: From local skin area *mg./min.*	Mean sweating rate: Mean flow per gland *µg./min.*
1	0.75	602	1.02	1.69
2	2.25	690	1.74	2.95
3	3.75	516	0.82	1.59
4	5.25	480	0.73	1.52

Figure 4 shows the results. The glands on the left forearm which had been kept inactive for the first 4.8 hours of the experiment showed a definite hypersecretion as compared to the glands on the right forearm that had continued to function throughout the entire heat exposure.

It was considered possible that repeated injections of atropine caused the glandular cells to become sensitized to the action of acetylcholine. This possibility was excluded by the finding that a skin area, pretreated by repeated intradermal injections of atropine and then allowed to rest for 45 minutes after the last injection of atropine, showed no greater response to intradermal Mecholyl than a control area which had received no pretreatment.

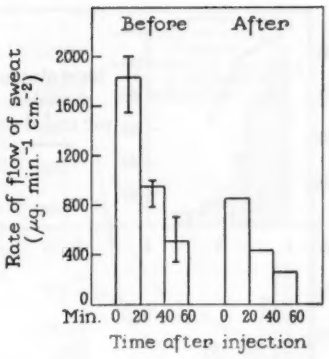

FIG. 3. THE REACTIVITY OF THE SWEAT GLANDS TO A STANDARD INTRADERMAL STIMULUS (β-METHYLACETYLCHOLINE HYDROCHLORIDE) UNDER RESTING CONDITIONS (LEFT) AND FOLLOWING 6 HOURS OF PROFUSE REFLEX SWEATING (RIGHT)

The columns to the left represent the mean of eight determinations; the vertical lines represent the range of variation.

Repeated intradermal injections or a constant intradermal infusion of Mecholyl evoked decreasing outputs of sweat until a refractory state was

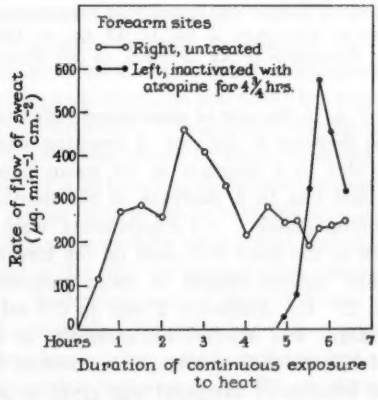

FIG. 4. COMPARISON BETWEEN THE FLOWS OF SWEAT FROM TWO AREAS OF SKIN

One area (right) has continued to function throughout a 6.8-hour heat exposure; the other area (left) has been kept inactive for the first 4.8 hours of the heat exposure by intradermal infiltration with atropine.

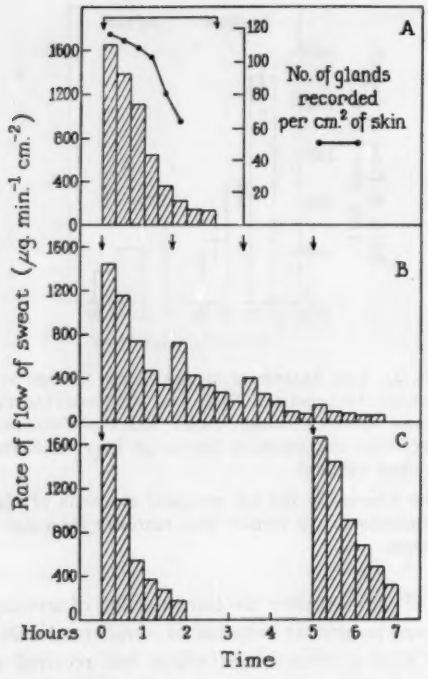

FIG. 5. A. THE FLOW OF SWEAT AND THE NUMBER OF FUNCTIONING GLANDS FOLLOWING A CONSTANT INTRADERMAL INFUSION OF MECHOLYL AT THE RATE OF 50 μg. PER MINUTE. B. THE FLOW OF SWEAT FOLLOWING FOUR INTRADERMAL INJECTIONS OF MECHOLYL, 2 MG. IN 0.5 ML. OF ISOTONIC SALINE, AT INTERVALS OF 100 MINUTES. C. THE FLOW OF SWEAT FOLLOWING TWO INTRADERMAL INJECTIONS OF MECHOLYL, 2 MG. IN 0.5 ML. OF ISOTONIC SALINE, SEPARATED BY AN INTERVAL OF 300 MINUTES

reached; as in the case of generalized reflex sweating the decrease in the rate of sweating was primarily due to a decrease in the mean flow per gland and less to a decrease in the number of functioning glands. All experiments were carried out at the same skin sites on the forearm of the same subject resting at room temperature, 20° to 25° C. Mecholyl, 2 mg. in 0.5 ml. isotonic saline, was injected intradermally at intervals of 100 and 300 minutes; or a constant intradermal infusion of Mecholyl was given at a rate of 50 μg. per minute. Collections of sweat and prints of the number of functioning glands were made in the usual manner. The results are shown in Figure 5. It will be seen that a full response to a second injection of Mecholyl was obtained when the interval between two injections was 5 hours (Figure 5C). When the interval was shorter than 5 hours each new injection resulted in a smaller response than the preceding one (Figure 5B). A very rapid decline in sweating rate was seen following the constant intradermal infusion (Figure 5A).

Sweat glands which had been rendered refractory to Mecholyl showed no response to the stimulus of general heat. Sweat collection chambers were glued to each of two sites on each forearm. At room temperature and with the subject at rest, four intradermal injections of Mecholyl were given at 40-minute intervals at each of the collection sites on the left forearm. Immediately thereafter the subject entered the hot room where he remained for a period of 80 minutes. The rates of sweating from all four sites were measured and compared.

The results, shown in Figure 6, demonstrate that the sweat glands on the left forearm, which had been rendered refractory to Mecholyl showed little response to the stimulus of general heat as compared to the untreated glands on the right forearm.

After a single injection of Mecholyl a period of 5 to 6 hours was required for complete restoration of glandular power. Sweat collection cham-

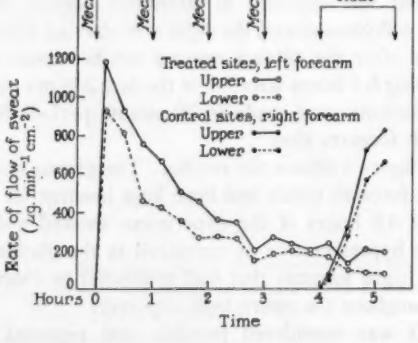

FIG. 6. COMPARISON OF THE RESPONSES TO GENERAL HEAT AT TWO SITES ON THE LEFT FOREARM, WHICH HAVE BEEN PRETREATED BY REPEATED INJECTIONS OF MECHOLYL, WITH THE RESPONSES AT SYMMETRICAL SITES ON THE RIGHT FOREARM WHICH HAVE REMAINED UNTREATED

bers were glued to two sites on each forearm. Mecholyl, 2 mg. in 0.5 ml. isotonic saline, was injected at all four sites and the rate of sweat secretion measured in six 20-minute periods. Injections of Mecholyl at all four sites were repeated after two hours and the rate of sweat secretion was again measured in six 20-minute periods. This experiment was repeated on each of the three following days, using the same four sites on the forearms but extending the intervals between the two injections of Mecholyl to 3 hours on the first day, 4 hours on the next and 5 hours on the last day. The total output of sweat in the six periods following the second injection of Mecholyl, Q_2, was compared to the total output of sweat in the six periods following the first injection of Mecholyl, Q_1. For the four sites the ratios, Q_2/Q_1, averaged 0.44, 0.76, 0.86, and 0.93 when the intervals between the first and second injection were 2, 3, 4, and 5 hours, respectively (Figure 7).

DISCUSSION

Apparently sweating can continue indefinitely at the same rate only as long as the demand upon the secretory mechanism does not exceed simultaneous processes that operate to restore secretory function. Whenever the demand upon the secretory mechanism exceeds restorative functions, the rate of sweating will decrease. This phenomenon is indicated in Figure 8. The curves show the characteristic decrease in sweating rate with time following each of two different intensities of stimulation with Mecholyl and following exposure to a hot environment. The constant intense stimulation of the sweat glands by intradermal infusion of Mecholyl is followed by more rapid diminishing of the rate of flow of sweat than the less intense stimulation by repetitive intradermal injections of Mecholyl or the still less intense reflex stimulation of heat. Following each of these different qualities of stimulation the sweating rate reaches an early maximum after which it decreases exponentially with a half time of 40, 90, and 180 minutes depending on the intensity of stimulation. A theoretical maximum rate of flow at which sweating may continue indefinitely was calculated by dividing the total output of sweat obtained following stimulation of the glands with a stand-

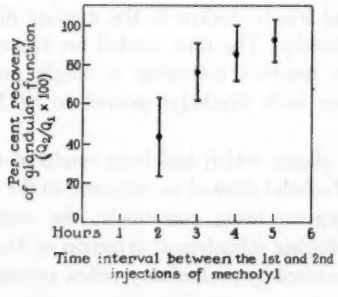

FIG. 7. THE RECOVERY OF GLANDULAR FUNCTION DETERMINED BY TWO IDENTICAL INJECTIONS OF MECHOLYL GIVEN 2, 3, 4, AND 5 HOURS APART

The total output of sweat following the second injection, Q_2, has been calculated as a percentage of the total output of sweat following the initial injection, Q_1. The points represent mean values determined from four sites; the vertical lines represent the corresponding standard deviations.

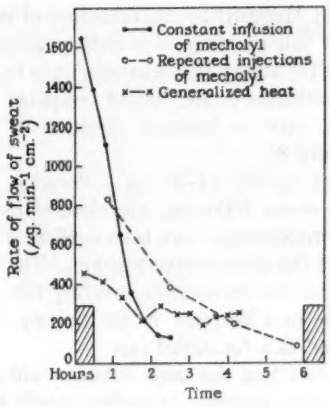

FIG. 8. THE DECLINE IN THE RATE OF SWEATING FOLLOWING A CONSTANT INTRADERMAL INFUSION OF MECHOLYL, REPEATED INJECTIONS OF MECHOLYL AT 100-MINUTE INTERVALS AND THE STIMULUS OF HEAT

The closed circles represent the sweating rates obtained during infusion of Mecholyl at the rate of 50 μg. per minute. The open circles represent mean sweating rates in each of the 100-minute periods in Figure 5B. The two bars represent a theoretical maximal rate at which sweating may continue indefinitely without evidence of fatigue (calculated from the mean total sweat output of the two 120-minute periods in Figure 5C divided by 300 minutes.)

ard dose of Mecholyl by the minimum of time required for full recovery of secretory function as judged by the ability of the same glands to reproduce quantitatively the initial response when challenged with an identical injection of Mecholyl (Figure 8).

Previous reports (1–3) on a decrease in the rate of sweating following a prolonged exposure to a hot environment, have been confirmed by the findings of the present investigation. The results indicate that the decrease in sweating rate is primarily due to a decrease in the activity of each sweat gland as a functional unit.

It is shown that the same stimulus will elicit a more vigorous response in resting glands than in glands that have been exhausted by previous function. This is true for the physiological stimulus of environmental heat (Figure 4) as well as for the pharmacological stimulus of a cholinergic drug (Figure 3). Likewise a decreased reactivity to the physiological thermal stimulus is seen as well in glands that have been exhausted by previous reflex sweating (Figures 1 and 4) as in glands that have been exhausted by intensive sweating due to repeated injections of cholinergic drug (Figures 5B and 6). These findings indicate that the decrease in sweating rate on prolonged reflex sweating is primarily due to an exhaustion of the secretory mechanism of the sweat gland, and not to a decrease in nervous stimulation. The fact that previously rested and previously functioning glands react differently to the same stimulus (Figure 4) excludes the possibility that the drop in sweating rate may be mediated by humoral factors.

Therefore, it seems reasonable to speak of "fatigue" of the sweat glands as the primary cause of the decrease in sweating rate noted on prolonged exposures to intense heat. The experiments with cholinergic stimulation suggest that the degree of fatigue of the glands becomes greater the more the demand upon the secretory mechanism exceeds the simultaneous restorative capacity. Thus fatigue of the sweat glands and defective restoration of secretory activity may well be the primary factors involved in the induction, perpetuation, and progression of human heat stroke.

SUMMARY

Earlier work has shown that the rate of sweat secretion decreased during prolonged exposures to environmental temperatures greater than 40° C. In the present investigation this observation has been confirmed and extended by a study of the activity of sweat glands in response to local chemical stimulation as well as to general heating of the body. A fall in sweating rate of from 30 to 60 per cent of maximum flow has been shown to occur during the third to fifth hour of exposure to an environment of 40° to 45° C., relative humidity 60 to 90 per cent. Quantitative collections of sweat and registrations of the number of functioning glands from the same area of skin indicate that this response is due to a decrease primarily in the activity of each glandular unit and secondarily in the number of functioning units. The relative activities[1] of the sweat glands have been shown to remain unchanged during exposure to heat, indicating that the absolute decline in function of any single gland is proportional to its original or maximal functional capacity.

Repeated intradermal injections or a constant infusion of β-methylacetylcholine hydrochloride, Mecholyl, evoked decreasing outputs of sweat until a refractory state was reached; as in the case of general reflex sweating the decrease in secretion rate was due chiefly to decline in the flow per gland, and less to decline in the number of functioning glands. The time needed for recovery of glandular function following a single maximal stimulation with Mecholyl proved to be 5 to 6 hours.

Sweat glands which had been rendered refractory to Mecholyl showed no response to the stimulus of general heat; conversely, the output of sweat following intradermal injection of Mecholyl was diminished by preliminary reflex sweating.

CONCLUSIONS

These results suggest that the decrease in reflex sweating produced by environmental heat is due to fatigue of the glandular units and not to decreased central nervous stimulation. Apparently sweating can continue indefinitely at the

same rate only if the demand on the secretory mechanism does not exceed simultaneous processes that operate to restore secretory function. When the demand upon the secretory mechanism exceeds the capacity for restoration of function, the sweating rate decreases.

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THE EFFECT OF POTASSIUM DEFICIENCY UPON ADRENOCORTICAL SECRETION IN THE RAT [1]

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(Submitted for publication July 11, 1955; accepted August 10, 1955)

The potassium-deficient rat exhibits several features in common with the untreated adrenalectomized rat, among which are subnormal growth, lowered blood pressure (1–3), diminished blood pressure responsiveness to pressor substances (4), and decreased tolerance to trauma. It therefore was considered important to determine whether the depressor response induced by potassium depletion might in fact be due to an associated state of adrenocortical insufficiency induced by the chronic potassium depletion. Such a mechanism appeared plausible particularly in view of our finding that the administration of cortisone rapidly returns the lowered blood pressures induced by potassium deficiency in rats to their initially normotensive (5) or hypertensive levels (6).

MATERIALS AND METHODS

A series of male rats (Long-Evans strain), initially aged 5 to 6 weeks, was used in this investigation. The rats were divided into three groups. One group of 6 rats (I) was fed a synthetic ration deficient in potassium, as previously described (2); this diet contained 0.004 per cent potassium and 1.4 per cent sodium.[2] A second group of 8 control rats (II) was fed the same synthetic ration but with added potassium chloride; it contained 0.52 per cent potassium and 1.4 per cent sodium.[2] For further control purposes a third group of 10 rats (III) was fed stock laboratory ration (containing 0.78 per cent potassium, 0.80 per cent sodium[2]). Seven weeks later the blood pressure of each rat was obtained with the microphonic manometer (7). Each rat was tail-bled for determination of plasma potassium 48 hours prior to collection of adrenal venous blood.

The secretion of adrenocortical steroids then was determined in all rats by the following technique. Under light ether anesthesia the rat was injected intramuscularly with 2 mg. sodium heparin, the abdominal cavity of the rat was opened and the right adrenal gland excised. The left kidney was "slipped" out of its capsule and removed after ligation of its vascular pedicle. A polyethylene cannula was introduced into the left renal vein through a small incision just proximal to the pedicle ligature and was ligated in place in such a manner that its bevelled distal tip approximated the left adrenal vein as the latter entered into the renal vein. Any accessory veins which emptied into the adrenal vein were then ligated. Another ligature was placed about the left renal vein at the juncture with the inferior vena cava. The cannula was brought out through a left lateral stab wound, the abdominal incision closed and the animal placed in a restraining cage. The adrenal venous blood was drained by gravity into a cold, heparinized test tube held in a chilled container filled with ice. In general, collection of blood was continued over a period of 60 to 120 minutes, during which 2.5 to 4.0 ml. of blood was obtained.

Each sample of adrenal venous blood was subjected to the following analysis for its steroid content. One to 2 ml. of plasma were extracted with 50 ml. of chloroform. The phenylhydrazine chromogen was extracted with 1 ml. reagent and developed overnight at room temperature according to the procedure of Silber and Porter (8). The curves of the reactive steroids were read between 320 and 450 mμ, using 0.5 ml. cuvettes in a Beckman Model DU spectrophotometer. From these data values were computed for steroids absorbing maximally at 350 and 410 mμ, by the system of Vickerstaff (9) for two component mixtures on the assumption (10, 11) that corticosterone (compound B) and 17-hydroxycorticosterone (compound F) are the main products of the adrenal effluent, other C_{19} and C_{21} steroids which have been detected by paper chromatography contributing less than 10 per cent of the total absorption at each of the two significant wave lengths, 350 and 410 mμ. The fact that for the rat, the extinction of 17,21-dihydroxy, 20-keto-steroids at 410 mμ is 5 times that of the predominating corticosterone at 350 mμ (8) and also that the absorption peaks are well separated, validates the use of this type of calculation. The amount of Silber-Porter chromogen of aldosterone is too small to be detected by this method, since aldosterone is secreted at a rate of only 0.2 to 0.8 μg per Kg. per hr. (11). Corticosterone (Merck)[3] was used as the standard for compounds with absorption maxima at 350 mμ and 17-hydroxycorticosterone (Merck) for those at 410 mμ. Most of the curves

[1] Aided by Grants from the American Heart Association, Life Insurance Medical Research Fund, San Francisco Heart Association and the National Institute of Health, Grant H-1006, Public Health Service.

[2] As determined by flame photometry of ashed diet.

[3] Provided by the courtesy of Dr. Frederick K. Heath of Merck & Co., Inc., Rahway, New Jersey.

TABLE I

Effect of potassium depletion on adrenocortical secretion of the rat

Group	Type of rat	Rat no.	Weight (gm.)	3-keto, Δ4, adrenal steroids calculated as corticosterone		17,21-dihydroxy, 20-keto, steroids calculated as 17-hydroxycorticosterone	
				Concentration (μg/ml. plasma)	Secretory rate (μg/Kg./hour)	Concentration (μg/ml. plasma)	Secretory rate (μg/Kg./hour)
I	Rats fed potassium-deficient diet	12A	180	16.2	96.9	2.2	13.4
		13A	220	15.6	180.0	2.0	22.5
		14A	250	30.0	305.0	2.7	28.0
		15A	240	25.0	229.0	1.2	11.0
		16A	196	16.2	186.2	1.5	17.3
		17A	230	33.7	84.7	4.3	10.8
	Average: 6		219	22.7	180.3	2.3	17.1
	Range:			(15.6–33.7)	(84.7–305.0)	(1.2–4.3)	(10.8–28.0)
II	Rats fed potassium-deficient diet with added KCl	36A	250	12.5	217	0.7	12.1
		37A	250	13.7	303	1.8	39.4
		38A	242	16.2	92	1.0	5.7
		39A	242	23.6	369	2.0	3.1
		40A	236	23.7	182	1.8	14.0
		41A	200	23.6	184	1.2	9.3
		42A	218	13.7	217	0.7	10.0
		46	240	17.5	178	2.0	21.0
	Average: 8		235	18.0	215	1.4	14.3
	Range:			(12.5–23.7)	(92–369)	(0.7–2.0)	(3.1–39.4)
III	Rats fed stock diet	3A	200	57.5	290	5.0	25.0
		6A	218	15.3	135	1.3	11.6
		7A	298	16.2	164	1.5	15.2
		8A	230	11.2	64	0.8	4.7
		47	212	30.0	129.3	3.1	13.2
		48	252	14.8	114.8	0.8	6.4
		49	308	6.2	56.9	0.6	5.6
		50	252	26.2	216.4	1.5	13.4
		51	238	26.2	168	1.8	11.3
		2A	220	30.0	266	4.2	37.4
	Average: 10		238	23.3	160.4	2.0	14.2
	Range:			(6.2–57.5)	(56.9–290)	(0.6–5.0)	(4.7–37.4)

of the derived values reported showed absorption maxima at 350 mμ, rarely a shift to 380 and 390 mμ was noted.

RESULTS

The individual and average values for the steroids obtained from the three groups of rats are shown in Table I. At that time the average blood pressure of the potassium-deficient rats was 84 mm. Hg (Range: 78 to 88) in contrast with the average blood pressure of 108 mm. Hg (Range: 94 to 120) in the control rats fed stock diet. The average plasma K of the deficient rats was 3.5 mEq. per L. (Range: 2.7 to 3.7) and that of the control rats fed stock diet was 5.6 mEq. per L. (Range: 5.1 to 6.6). Elsewhere (3, 5, 12) we have shown that muscle potassium depletion also is present in such rats. The results are presented in terms of the concentrations (μg per ml. plasma) and rates of secretion (μg per Kg. weight per hour) of 3 keto, Δ4, adrenal corticosteroids calculated as corticosterone (compound B) and of 17,21,hydroxy, 20-keto-corticosteroids calculated as 17-hydroxycorticosterone (compound F).

As can be noted in Table I, the potassium-deficient rat as well as the normal animal is capable of putting out considerable amounts of steroids, the deficient animal averaging 180.3 μg per Kg. per hr. and the control rat, 160.4 μg per Kg. per hr. Since the concentrations of corticosterone in the plasma of the two groups are quite similar (22.7 and 23.3 μg per ml. plasma), this slight difference in secretory rates may be accounted for on the basis of size, the deficient animals being slightly smaller. In general, corticosterone was

found to be secreted in a tenfold greater concentration than was 17-hydroxycorticosterone. The concentrations and rates of secretion of 17-hydroxycorticosterone in the potassium-deficient rats were of the same magnitude as in the control rats.

It can be noted that considerable variation occurred in the plasma concentrations and rates of secretion of both steroids. This seems to be characteristic of the adrenal secretion of various animals tested by present day methods (10, 13–15). However, with only an occasional exception, the range of values was generally similar in all three groups of animals. Considering the relatively small sampling, the average values observed in the three groups also appeared to be of similar magnitudes.

DISCUSSION

Previous studies from this laboratory have shown that dietary potassium deprivation induces a fall of blood pressure in normotensive (1) and hypertensive (2, 3) rats which is a specific effect of potassium depletion (16) and which occurs in association with a somewhat diminished blood pressure responsiveness to pressor substances (4). Administration of potassium to such rats rapidly restores their blood pressure responsiveness and their blood pressures (16), if the adrenals are intact (12). We have considered (17) the possible role of insufficiency of adrenocortical secretion in this depressor response to potassium deficiency because of these and other common features of the potassium-deficient and the untreated, adrenalectomized rat. That potassium depletion might induce a hypotensive response by suppressing adrenocortical function also was suggested by the fact that potassium deficiency evokes a chronic alarm reaction (18) as well as by our earlier observation (5, 6) that cortisone promptly restores the lowered blood pressures of potassium-deficient rats to their respective normotensive or hypertensive levels without altering their potassium-depleted state.

On the other hand, some of the responses to potassium deficiency are inconsistent with a pattern of adrenocortical insufficiency. Thus, there is considerable indirect evidence that potassium deficiency stimulates production of adrenocorticoids (18–20) by evoking a chronic "alarm reaction" (18). Enlargement of the adrenals occurs despite inactivation of the glomerulosa zones, and the adrenals of such rats are depleted of ascorbic acid (18, 19, 21). Furthermore, such rats also exhibit increased liver glycogen and diminished glucose tolerance (18, 20), involution of the thymus (19, 20) and decreased circulating blood eosinophils and lymphocytes (18, 19). Although potassium deficiency appears to suppress the secretion of aldosterone (11), on the basis of present evidence it seems unlikely that the depressor response might be ascribed to this, since administration of the analogous hormone, DCA, is without pressor effect in potassium-deficient rats and indeed has a depressor and toxic effect in such animals (22).

We have attempted to clarify the relationship of potassium deficiency and adrenocortical function by direct determination of adrenal secretion in potassium-deficient rats. The values recorded for our control rats are in good agreement with those recorded by Bush (10) and by Singer and Stack-Dunne (11), despite the use of a completely different method of analysis. The data in this study indicate essentially similar averages and ranges of concentration and rates of secretion of corticosterone and 17-hydroxycorticosterone by the potassium-deficient rats, when compared to the control animals. It is important to point out that the technique employed for collection of adrenal venous blood in this study probably constitutes a maximal stimulus for adrenocortical secretion. Under these circumstances, however, the potassium-deficient hypotensive rat appeared to respond in a fashion similar to that of the control animals. It is of interest that Singer and Stack-Dunne (11) also recently found that potassium deficiency fails to affect corticosterone secretion in rats. Therefore, it must tentatively be suggested that the hypotensive response induced by potassium deficiency is not mediated by supression of adrenocortical secretion. This conclusion appears justified in view of the present data and the fact that trauma comparable to the operative procedure used here fails to raise the lowered blood pressure of the potassium-deficient rat.

SUMMARY

The effect of chronic potassium deficiency upon adrenocortical steroid secretion was studied in

rats. The data indicate that, under the stress of the experimental method, the rates of secretion of corticosterone and 17-hydroxycorticosterone by potassium-deficient rats are essentially similar to those of control animals. It is concluded that the hypotensive response induced by potassium depletion is not mediated by a suppression of adrenocortical secretion of these steroids.

ACKNOWLEDGMENT

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GLUCAGON-INDUCED HYPERGLYCEMIA AS AN INDEX OF LIVER FUNCTION [1, 2]

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Glucagon is a protein substance in pancreatic extracts which raises blood glucose by stimulating hepatic glycogenolysis (1–3). For this reason, it has been termed the hyperglycemic glycogenolytic factor (HGF) of the pancreas. Sutherland and Cori (2) have shown that glucagon increases the concentration of active phosphorylase in liver slice preparations. Apparently, glucagon administration does not induce glycogenolysis in muscle (4, 5).

Whether glucagon has an effect on extrahepatic utilization of glucose is still a matter of controversy. Drury, Wick, and Sherrill (6) have reported that the rate of uptake of C^{14}-labeled glucose by eviscerated-nephrectomized rabbits actually decreases when glucagon is administered to such animals. On the other hand, Root, Ellis, and Staub (7), using intact rabbits and mice, have demonstrated that the presence of glucagon in insulin solutions in ratios of from 1:100 to 1:1 does not influence the degree of hypoglycemia produced by insulin and does not alter the potency of the insulin preparation as determined by the mouse convulsion assay. Ingle, Nezamis, and Humphrey (8) were unable to demonstrate any influence of glucagon upon extrahepatic glucose utilization in eviscerated rats. Using peripheral arteriovenous glucose differences as an index of peripheral glucose utilization in healthy human subjects, Van Itallie, Morgan, and Dotti (9) have found that glucagon does not inhibit and may accelerate uptake of glucose by peripheral tissues.

It has been known for many years that the diseased liver usually does not contain a normal quantity of glycogen. Brill and Fitz-Hugh (10) and Loeb, Reeves, and Glasier (11) reported that the hyperglycemic response to a standard dose of epinephrine in patients with hepatic disease is well below normal. However, injection of epinephrine has serious disadvantages as a means of estimating liver glycogen content. In doses needed to produce a maximal hyperglycemic response (1.0 mg. or more), epinephrine administration frequently is associated with such side effects as headache, dizziness, and palpitation. Cardiac arrhythmias and cerebral hemorrhage, although rare complications, have occurred following epinephrine injection (12). Because of its potential toxicity epinephrine is almost always administered subcutaneously or intramuscularly, and the rate at which it enters the circulation from such sites is necessarily variable. Thus, the time at which its maximal hyperglycemic effect can be measured is uncertain.

In contrast, glucagon exerts a more potent glycogenolytic effect on liver, on a molecular basis, than does epinephrine (13), and is without reported toxic side effects, even when administered intravenously in large amounts. Because of these apparent advantages of glucagon over epinephrine, an attempt has been made to determine whether glucagon-induced hyperglycemia constitutes a potentially useful index of liver function.

PROCEDURE

Ten healthy graduate students and eight patients with severe parenchymal liver disease served as subjects. All subjects were in the postabsorptive state at the time of study. Diagnoses of the patients and results of liver function tests are in Table I. The glucagon (Lilly No. 208-158B-214)[4] used in the study was prepared by the method of Staub, Sinn, and Behrens (14). It was highly purified, containing only 0.005 to 0.05 unit of insulin per mg. glucagon. The material employed was of uniform potency.

The glucagon was administered intravenously in approximately 150 ml. isotonic sodium chloride solution at

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[4] Supplied through the courtesy of Dr. W. R. Kirtley of Eli Lilly and Company, Indianapolis, Indiana.

TABLE I

Patients with hepatic disease: Results of liver function tests

Patient	Diagnosis	Thymol turbidity (*units*)	Cephalin flocculation	Alkaline phosphatase (*Bodansky units*)	Serum bilirubin (*mg. per 100 ml.*)	Serum albumin (*gm. per 100 ml.*)	Serum globulin (*gm. per 100 ml.*)	Bromsulfalein retention	
								15 min.	45 min.
								%	%
H. R.	Laennec's cirrhosis of liver	13.7	++++	7.6	4.0	3.8	4.9	63	40
G. F.	Laennec's cirrhosis of liver	4.9	++++		0.4	4.4	2.9		23
E. E.	Laennec's cirrhosis of liver	5.7	++	15.1	39.2	2.8	6.6		
A. G.	Homologous serum hepatitis	9.7	++++	7.1	17.3	4.2	3.3	21	10
J. F.	Laennec's cirrhosis of liver	2.6	++++	5.7	5.6	2.8	4.1	49	21
H. C.	Infectious hepatitis	4.4	++++	10.6	12.8	5.4	3.9	32	13
A. C.	Laennec's cirrhosis of liver	4.9	++++	12.0	3.6	2.7	3.9	61	38
M. G.	Laennec's cirrhosis of liver	6.9	0	7.2	3.6	4.1	3.8	64	46

a constant rate by means of a Bowman constant infusion pump. Duration of infusions was approximately 30 minutes.[5] The dose of glucagon in every instance was 0.03 mg. per Kg. body weight.

In the normal subjects, control capillary and venous blood samples were obtained virtually simultaneously immediately prior to glucagon administration, at the end of glucagon infusion, and at 25-minute intervals for approximately 75 minutes thereafter. In patients with liver disease the same schedule was followed except that capillary blood samples were not taken.

On a second occasion, the experimental procedure was repeated in all normal subjects and patients except that synthetic epinephrine (Suprarenin®) [6] was administered subcutaneously 10 minutes prior to glucagon infusion. The dose of epinephrine was 0.003 mg. per Kg. body weight. In such instances, control glucose samples were obtained immediately prior to epinephrine administration.

In a third series of experiments, six of the normal subjects were given 0.003 mg. per Kg. synthetic epinephrine subcutaneously. In this group, capillary and venous glucose levels were determined before and at frequent intervals for approximately 100 minutes following epinephrine injection.

For three days prior to each experiment, each subject was kept on a diet providing at least 250 gm. of carbohydrate per day (15). Glucose in whole blood was measured by the method of Somogyi (16, 17), as modified by Nelson (18). Venous blood samples were obtained with a minimum of stasis from an antecubital vein, and almost simultaneously, capillary blood (which has the same glucose concentration as arterial blood [19, 20]) was collected by direct pipetting from a finger tip of the same extremity after cutaneous puncture. Determinations were done in duplicate on 0.2 ml. samples.

To permit comparison of peripheral glucose uptakes in the presence of differing capillary glucose levels, relative assimilation indexes (R.A.I.) were calculated for each experiment in which capillary-venous glucose differences were measured, according to the formula of Somogyi (21):

$$\text{R.A.I.} \frac{100 \times \Sigma(C - V)}{\Sigma(i)},$$

where

$\Sigma(C - V)$ = assimilation index = sum of the capillary venous differences

$\Sigma(i)$ = hyperglycemic sum = sum of the increments in capillary blood glucose over the control level during the corresponding time interval.

(Negative increments and their corresponding C-V differences were omitted from the calculations and negative C-V differences associated with positive increments were assigned a value of zero.)

RESULTS

A typical hyperglycemic response to intravenous glucagon in a normal subject is shown in part A of Figure 1. The capillary and venous glucose values rise rapidly during glucagon infusion and fall rapidly after the end of glucagon administration to levels below initial, control values. Appreciable capillary-venous (C-V) differences are maintained on the falling segment of the curve, indicating removal of glucose by peripheral tissues.

[5] Later studies have shown that prolonged injection of glucagon is not necessary. Comparable hyperglycemic responses can be induced when the same amount of glucagon diluted in 10 ml. saline is injected rapidly by syringe.

[6] Supplied through the courtesy of Dr. A. Scribner, Winthrop-Stearns, Inc., New York, New York.

In part B of Figure 1 is shown the modification of the capillary-venous glucose pattern which occurs when 0.003 mg. per Kg. synthetic epinephrine is administered subcutaneously 10 minutes prior to intravenous administration of the same quantity of glucagon to the same subject for approximately the same time interval. In this instance, the glucagon-induced hyperglycemia is augmented and the venous and capillary glucose levels at all points of the curve are close together.

In part C of Figure 1 is shown the hyperglycemic response of the same individual to 0.003 mg. per Kg. synthetic epinephrine given subcutaneously. Capillary-venous glucose differences remain negligible throughout.

Capillary and venous increments over control values in the normal subjects following administration of (a) glucagon alone, (b) epinephrine and glucagon together, and (c) epinephrine alone, are given in Table II. The relative assimilation indexes (R.A.I.) calculated for each subject are also in Table II.

Comparison of the means of the relative assimilation indexes shows that epinephrine administration, either alone or in combination with glucagon is associated with a significantly lower index of glucose assimilation than that which occurs when glucagon alone is administered ($p < 0.01$).

Venous increments over control values in the patients with liver disease following administration of (a) glucagon, and (b) glucagon and epinephrine conjointly, are given in Table III. In contrast to the findings in the normal subjects, use of epinephrine in conjunction with glucagon in the patients with liver disease does not result in a greater mean rise in blood glucose than that induced by glucagon administration alone.

In Figure 2, the means of the hyperglycemic responses (venous blood) to glucagon alone of the normal subjects and of the patients with liver diseases are compared. The configuration of the curve representing the mean response of the normal group is similar to that displayed by the single normal subject depicted in Figure 1A. Again, the appreciable secondary hypoglycemia following the hyperglycemic phase is evident.

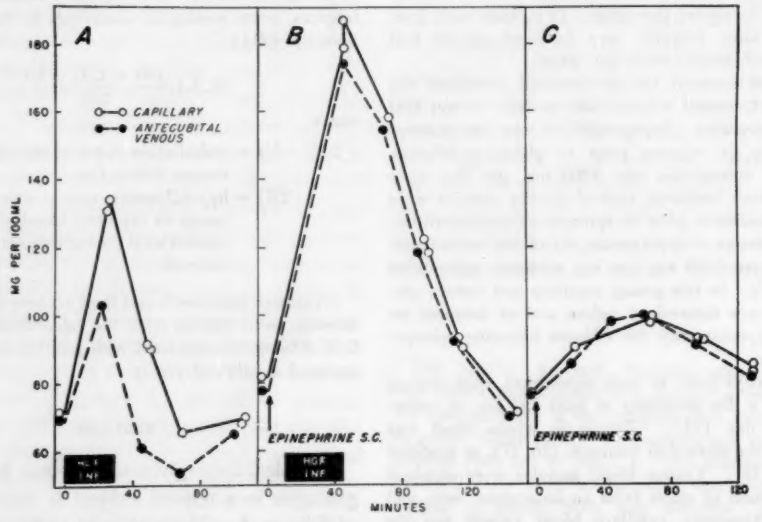

FIG. 1. (SUBJECT J. G.) A. GLUCAGON-INDUCED HYPERGLYCEMIA IN A NORMAL SUBJECT, WITH LARGE CAPILLARY-VENOUS (C–V) DIFFERENCES MAINTAINED ON THE FALLING LIMB OF THE CURVE (2.1 MG. OF GLUCAGON IN SALINE GIVEN BY CONSTANT INFUSION). B. ACCENTUATION OF GLUCAGON-INDUCED HYPERGLYCEMIA AND DIMINUTION OF C–V DIFFERENCES DURING ADMINISTRATION OF 2.1 MG. GLUCAGON BY CONSTANT INFUSION PRECEDED BY SUBCUTANEOUS INJECTION OF 0.20 MG. OF EPINEPHRINE. C. EFFECT ON GLUCOSE LEVELS AND PATTERN OF C–V DIFFERENCES OF 0.20 MG. EPINEPHRINE INJECTED SUBCUTANEOUSLY

TABLE II

Effect of glucagon alone, glucagon and epinephrine together, and epinephrine alone on capillary and venous glucose levels in normal subjects

Subject	Minutes after start of glucagon administration (duration of infusion 30 minutes)								Relative assimilation index (RAI)
	30		55		80		105		
	C*	V†	C*	V†	C*	V†	C*	V†	
Subjects given glucagon intravenously (0.03 mg./Kg. body weight)									
J. H.	35	29	25	− 2	−18	−27	−12	−14	61.7
J. S.	64	38	31	7	−16	−21	−25	−30	54.7
N. D.	39	22	−19	−20	−26	−28	− 8	−10	46.2
F. C.	46	31	14	5	− 9	−11	—	—	40.0
R. B.	74	55	71	63	15	13	—	—	23.8
H. S.	41	14	2	−26	−32	−41	−20	−24	132.6
D. H.	59	54	11	0	−24	−26	−21	−18	37.1
A. K.	55	40	34	22	− 1	− 6	−13	−14	32.6
A. B.	60	34	47	23	−23	−24	−22	−23	44.9
J. G.	53	33	17	−11	− 7	−14	− 4	− 5	74.3
						Mean and Standard Deviation			54.8±31.0
Subjects given glucagon intravenously (0.03 mg./Kg. body weight) after subcutaneous injection of epinephrine (0.003 mg./Kg. body weight)									
J. H.	59	64	66	74	11	10	−20	−14	8.8
J. S.	84	82	92	92	36	35	0	1	9.4
N. D.	63	64	73	65	20	11	− 2	− 5	17.9
F. C.	78	80	110	111	82	86	60	65	2.1
R. B.	77	69	96	88	64	55	23	23	9.6
H. S.	58	57	59	61	18	22	0	4	14.1
D. H.	88	75	120	111	80	75	38	35	10.4
A. K.	68	70	89	93	67	71	—	—	0.9
A. B.	76	73	86	84	− 2	− 4	−31	−34	13.0
J. G.	96	96	76	74	36	35	6	8	7.9
						Mean and Standard Deviation			9.4±5.1
Subjects given epinephrine subcutaneously (0.003 mg./Kg. body weight)									
J. H.	17	14	22	20	11	9	4	2	16.7
J. S.	20	18	32	31	18	19	6	5	14.5
N. D.	19	16	20	18	10	8	5	4	14.8
F. C.	24	25	23	19	12	10	9	3	22.1
R. B.	17	23	14	21	3	9	0	5	11.8
J. G.	14	10	34	32	33	31	16	14	10.3
						Mean and Standard Deviation			15.0±4.1

* Capillary glucose increments over control value, in mg. per 100 ml.
† Venous glucose increments over control value, in mg. per 100 ml.

The over-all response of the patients with liver disease is discernibly different. The slower rise, the somewhat lower, late peak, and the slower descent of the curve all are distinguishing features. Although the curves have different configurations, consideration of the standard deviations of the individual means shows that there is considerable overlap between the two groups.

The hyperglycemic responses (venous) of the same normal subjects and the same patients with liver disease given the same quantity of glucagon at the same rate but following pre-treatment with epinephrine are compared in Figure 3. By this modification of the procedure, the overlap between the patients with liver disease and the normal subjects has been eliminated and the difference between the responses of the two groups becomes striking.

When glucagon alone is administered (Figure 2) the difference between the means of the responses (venous blood glucose) of normal subjects and patients with hepatic disease enters the 0.01 range of statistical significance only at one point, 50 minutes after cessation of glucagon infusion. When glucagon and epinephrine are administered together (Figure 3) the differences between the normal group and the liver-disease group at the end of the glucagon infusion and at the peak of the curve are of a high order of statistical significance ($p < 0.001$).

TABLE III

Effect of glucagon alone and glucagon and epinephrine together on venous glucose levels in patients with liver disease

Patient	Minutes after start of glucagon administration (duration of infusion 30 minutes) 30 V*	55 V*	80 V*	105 V*
Patients given glucagon intravenously (0.03 mg./Kg. body weight)				
H. R.	9	19	21	14
G. F.	21	41		
M. G.	43	40	17	− 7
A. C.	15	21	13	4
E. E.	13	11	6	− 1
H. C.	23	24	−2	−15
A. G.	4	2	−3	− 8
J. F.	17	18	−3	−25
Patients given glucagon intravenously (0.03 mg./Kg. body weight) after subcutaneous injection of epinephrine (0.003 mg./Kg. body weight)				
H. R.	14	29	41	
G. F.	21	42		
M. G.	40	53	36	19
A. C.	24	37	28	10
E. E.	4	5	6	7
H. C.	30	32	8	−25
A. G.	9	11	2	− 5
J. F.	13	8	6	5

* Venous glucose increments over control values, in mg. per 100 ml.

DISCUSSION

When glucagon alone is given to normal subjects, the hyperglycemic responses which ensue are difficult to interpret without consideration of capillary as well as venous glucose values. Reference to Figure 1A and Table II indicates that during and immediately after glucagon administration extrahepatic utilization of the released glucose is continuing at a rapid rate. Hence, it is difficult by means of isolated determinations of venous glucose values to know whether the observed glycemic level is low in a given instance because of rapid glucose uptake by peripheral cells, or because the ability of the liver to store or release glycogen is impaired.[7] Although more information about these alternative possibilities can be gained from simultaneous determination of capillary and venous glucose in an extremity, serial measurements of both capillary and venous glucose levels are not feasible as a routine clinical procedure.

[7] It is possible that in the cirrhotic liver vascular shunts (*22*) may of themselves modify the hyperglycemic response to such agents as glucagon and epinephrine.

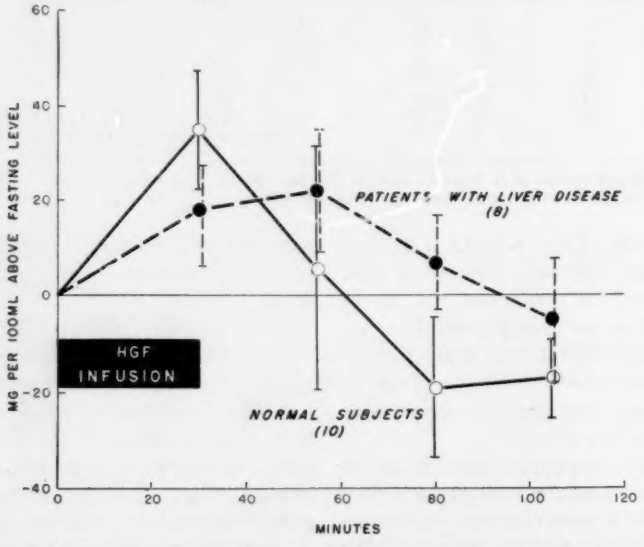

FIG. 2. COMPARISON OF MEANS OF HYPERGLYCEMIC RESPONSES (VENOUS) OF NORMAL SUBJECTS AND OF PATIENTS WITH LIVER DISEASE TO GLUCAGON ALONE

Vertical bars represent one standard deviation.

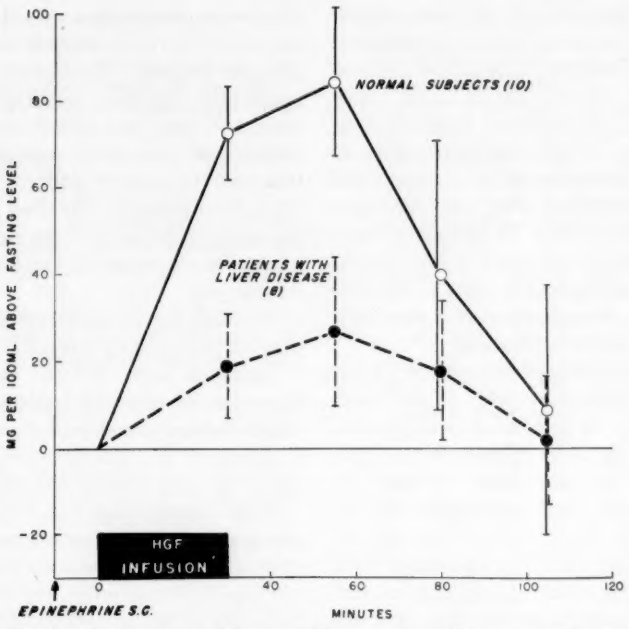

FIG. 3. COMPARISON OF MEANS OF HYPERGLYCEMIC RESPONSES (VENOUS) OF NORMAL SUBJECTS AND PATIENTS WITH LIVER DISEASE TO GLUCAGON AND EPINEPHRINE TOGETHER

Vertical bars represent one standard deviation.

Comparison of the hyperglycemic responses to glucagon of normal subjects and liver patients (Figure 2 and Tables II and III) indicates that although the mean curves of the two groups differ in pattern, the differences between the means approach statistical significance only at two points. In view of the severity of the liver damage in the patient group such differences cannot be regarded as satisfactory since patients with moderate or minimal hepatic disease could be expected to show even less distinctive responses to glucagon. Hence, glucagon-induced hyperglycemia, as such, does not appear to be a useful index of liver function.

The peripheral uptake studies performed on the normal subjects during and after glucagon administration indicate that the venous glucose values which occur during the hyperglycemic phase of the response to glucagon represent a resultant of two activities taking place simultaneously. These are (a) release of glucose from the liver, and (b) uptake of glucose by extrahepatic cells. Previous studies by the Coris (23), and others (21, 24, 25), have suggested that epinephrine acts to inhibit glucose uptake by peripheral cells. Hence, in a second series of experiments, epinephrine was administered in conjunction with glucagon to determine whether, by this means, the complicating variable of peripheral glucose utilization might be minimized. That this was accomplished is indicated by the greatly diminished relative assimilation indexes associated with administration of epinephrine along with glucagon (Table II). The modifying effect of epinephrine upon the pattern of C-V differences of glucagon-induced hyperglycemia is shown in part B of Figure 1.[8]

[8] If, as postulated, epinephrine has an inhibitory effect on peripheral utilization of glucose, one would expect that the hyperglycemic peaks obtained in normal subjects when glucagon and epinephrine are given together would be higher than could be accounted for by simple addition of the hyperglycemic responses of the same subjects to glucagon and epinephrine individually. (Even the assumption that there is an additive effect may be unwarranted [2].) Actually, in five of the six normal subjects whose responses to glucagon alone, glucagon and epinephrine together and epinephrine alone were

Comparison of the normal and liver-disease groups in terms of their responses to the glucagon and epinephrine combination (Figure 3) discloses differences between the two sets of means which attain a high order of statistical significance at the end of glucagon infusion and at the peak of the curve. Thus, administration of a small (nontoxic) dose of epinephrine along with glucagon reduces the modifying effect of peripheral utilization upon the venous and capillary glucose levels and permits a far better distinction to be made between the response to glucagon of normal subjects and patients with liver disease.

Although glucagon-induced hyperglycemia *per se* is clearly not a satisfactory index of liver function, measurement of the peripheral hyperglycemia induced by a combination of epinephrine and glucagon appears to be the best clinical method available (short of hepatic vein catheterization) for estimating the ability of the liver to store and release glycogen. Therefore, it has potential value as a test of liver function. Loeb, Reeves, and Glasier (11) have pointed out the lack of specificity of the hyperglycemic response to epinephrine, noting that patients with such "non-hepatic" disorders as carcinoma of the pancreas and diabetes mellitus also display an abnormally low rise in blood glucose following epinephrine administration. However, these authors did not record carbohydrate intakes prior to the time the epinephrine test was performed. Control of this dietary factor presumably would enhance specificity of the hyperglycemic response induced by such agents as epinephrine and glucagon.

Whether glucagon-epinephrine hyperglycemia will provide useful information about liver function which is not readily obtainable by means of existing tests now in wide use can be determined only after prolonged and detailed comparative studies.

SUMMARY AND CONCLUSIONS

Highly purified glucagon was administered intravenously to ten normal subjects and eight patients with parenchymal liver disease. Blood glucose levels were measured before, during, and after glucagon infusion. On a second occasion, the experimental procedure was repeated in the same normal subjects and patients except that a small quantity of epinephrine was administered subcutaneously 10 minutes prior to glucagon infusion. On a third occasion, the effect of the same quantity of epinephrine alone on capillary and venous glucose levels in six of the normal subjects was determined.

Although the curves representing the mean hyperglycemic response (venous) of the two groups to glucagon alone differed appreciably in configuration, the standard deviations of the individual means showed considerable overlap. Accordingly, it is believed that glucagon-induced hyperglycemia *per se* has little value as an index of liver function.

When epinephrine was administered prior to glucagon infusion in an attempt to minimize the complicating effect of extrahepatic glucose removal on venous glucose levels, the overlap between the responses of the normal subjects and the patients with hepatic disease was removed and the difference between the two groups became striking. Thus, it appears that glucagon-epinephrine-induced hyperglycemia has potential value as an index of one aspect of liver function.

ACKNOWLEDGMENT

The authors wish to thank Dr. Mary C. Morgan and Dr. Louis B. Dotti for their valuable assistance in carrying out these studies.

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studied, the glucagon-epinephrine-induced hyperglycemia was higher, usually by a considerable margin, than the sum of the hyperglycemias, respectively, induced by epinephrine alone and glucagon alone. This finding provides further support for the view that epinephrine has an inhibitory effect on peripheral glucose utilization.

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THE OCCURRENCE OF ACID MUCOPOLYSACCHARIDES IN HUMAN LEUKOCYTES AND URINE [1]

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Acid mucopolysaccharides have been found in urine (1) but have not been consistently demonstrated in the blood of normal subjects. Bassiouni (2) recently isolated a substance from leukocytes which he noted to be similar to chondroitin sulfate. In our laboratory leukocytes have been found to contain appreciable quantities of a substance resembling chondroitin sulfate. A direct relationship between this substance and the similar substance found in the urine has not been established.

METHODS

Subjects. Human subjects were used. These were hospital and laboratory personnel and patients who were unselected except for the omission of individuals with primary hematologic, metabolic, or rheumatic diseases.

Preparation of leukocytes. Siliconed needles, syringes, and glassware were used throughout the preparation until the step in which the cells were deliberately disintegrated. Venous blood (40 to 70 ml.) was drawn and distributed immediately in 10-ml. amounts to tubes containing 1.0 ml. of 5 per cent sodium citrate in a balanced salt solution.[2] The red blood cells were sedimented rapidly by mixing 2.5 ml. of 5 per cent dextran with the blood in each tube.[3] The supernatant leukocyte-platelet suspension in plasma was centrifuged at 85 g for 7 minutes, and the sedimented leukocytes were washed gently one time in 11.0 ml. of physiologic saline. Following recentrifuging, they were then pooled in 20 ml. of saline, counted, made up to 25 ml. with saline in process of transferring to a Raytheon Sonic Oscillator, and oscillated at 9 Kc. per second for 8 minutes.

Isolation of acid mucopolysaccharides from leukocytes. The oscillated material was made up to 50 ml. with saline, and 3 ml. of an aqueous mixed salt solution [4] were added. Acid mucopolysaccharides were then separated by the method of Astrup (3).[5]

Isolation of acid mucopolysaccharides from urine was accomplished as described in (1), except that collections of urine were made over a standard 3-hour period, samples were dialyzed before fractionation, and an aqueous mixed salt solution (17 per cent NaCl, 1.5 per cent Na_2SO_4) was added to each sample before fractionation (4).

Quantitative studies of acid mucopolysaccharides. On most samples of blood either a hexuronic acid or hexosamine determination was done. When possible both measurements were made on the same blood. The hexuronic acid content of the Astrup fraction was determined as in previous studies (1) by acid hydrolysis and production of the carbazole color reaction of Dische (5). The hexosamine content was determined by the method of Elson and Morgan (6), using redistilled acetylacetone and recrystallized p-dimethylaminobenzaldehyde. Prior hydrolysis of each sample for hexosamine determination was carried out by heating 3 ml. of sample with 1 ml. of 12 N hydrochloric acid in a sealed ampule for 15 hours in a 100° C. steam bath. The samples were then carefully neutralized with NaOH prior to determination of hexosamine content.[6] Standards of 40, 60, and 80 gamma of n-acetylglucosamine per ml. were similarly hydrolyzed and run as a standard curve with each set of determinations.

Qualitative studies of the acid mucopolysaccharides. Chromatographic studies were carried out by the same method as has been described previously (1) in studying urine fractions. Electrophoretic studies were done by the method of Pasternak and Kent (7), using versene-washed Whatman No. 1 paper, barbiturate buffer (pH 8.5, 0.06 M), and 0.8 ma, 105 volts for 5 hours. The leukocyte and urine samples were prepared for chromatography and electrophoresis as follows: The Astrup fraction was dialyzed against 20 volumes of distilled water with constant rotation for 15 hours at 5° C. The contents of the dialysate casing were then dried

[1] This work was supported in part by research grants from the National Institute of Arthritis and Metabolic Diseases of the National Institutes of Health, Public Health Service, the Duke University Research Council, the American Heart Association, the North Carolina Heart Association and the Baxter Laboratories, Inc.

[2] Ten-ml. stock solution (7.5 per cent NaCl, 0.75 per cent KCl, 0.1 per cent Na_2HPO_4, 0.12 per cent KH_2PO_4, 0.05 per cent K_2HPO_4) and 7 ml. 1 per cent Na_2HPO_4 per 100 ml., aqueous.

[3] Salt free Dextran-Benger Batch 3526/1, made up to final salt concentration of the balanced salt solution described.

[4] 17 per cent NaCl, 3.5 per cent NaH_2PO_4, 1.5 per cent Na_2SO_4, 3 per cent KCl.

[5] The filtration step after separation with ether was omitted.

[6] This step was accomplished by neutralization of an aliquot with 0.3 N NaOH, then neutralization of the test sample with 3 N NaOH, to avoid excessive dilution of the sample.

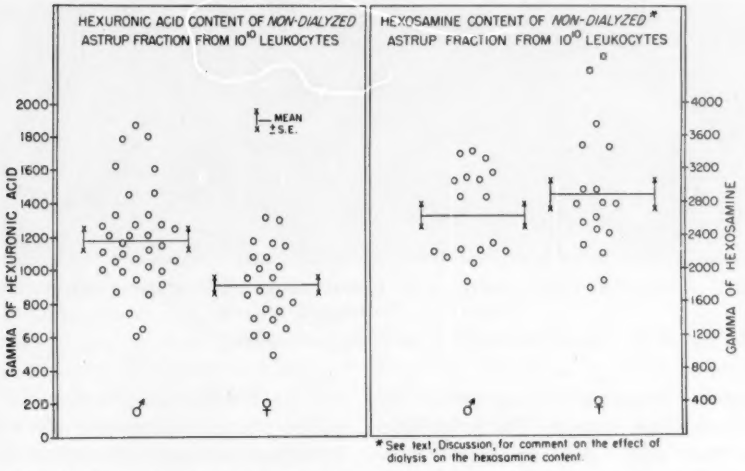

FIG. 1. HEXURONIC ACID AND HEXOSAMINE YIELDS FROM NON-DIALYZED ASTRUP FRACTION OF HUMAN LEUKOCYTES

rapidly in a Virtis Freeze-Dryer and taken up in 0.1 to 0.2 ml. of distilled water. On filter paper the material stained metachromatically with toluidine blue. Chromatographic and electrophoretic papers were stained with toluidine blue (8). Chondroitin sulfate (General Biochemicals, Inc.) and heparin (Liquaemin; Organon, Inc.) were used for comparison with the test samples.

RESULTS

Quantitative data for leukocytes prior to dialysis of the Astrup fraction are summarized in Figure 1. Data are expressed as gamma of hexuronic acid or of hexosamine per 10^{10} leukocytes (a quantity said by Rossiter and Wong (9) to have a wet weight of about 4 grams).[7] An Astrup fraction sample representing the residual sample pooled from many donors (male and female) was analyzed before and after dialysis of the Astrup fraction (Table I). It is apparent that the hexuronic acid content of the Astrup fraction was unaffected by dialysis. However, the relatively high hexosamine content was significantly reduced by dialysis. Using the calculated values of Dziewiatkowski (10) of 37.3 per cent uronic acid and 34.4 per cent hexosamine in sodium chondroitin sulfate, the hexosamine content after dialysis was twice the expected value for the amount of hexuronic acid present.

Figure 2 illustrates chromatograms comparing acid mucopolysaccharides of commercial source with those of human origin isolated from urine and leukocytes. It will be noted that the urine fraction shows inhomogeneity, as does the commercial preparation of chondroitin sulfate, with the major component of each exhibiting the same R_f value as does the single spot given in this range by the leukocyte fraction. The leukocyte fraction shows in addition some material remaining at the starting line, unlike the urine fraction. This material tended to stain orthochromatically with toluidine blue and could be moved out from the starting line with other solvent systems. It is at present unidentified. Residual material was not evident with the leukocyte fraction on paper electrophoresis (Figure 3), the entire fraction moving to form

TABLE I

Content of hexosamine and hexuronic acid in non-dialyzed and dialyzed samples of pooled Astrup fractions derived from leukocytes of human males and females

	Gamma per ml. of pooled Astrup fraction	
	Hexosamine	Hexuronic acid
Non-dialyzed	19.37	8.58
Dialyzed	15.56	8.89

[7] The amount present per sample analyzed was usually of the order of magnitude of 10 gamma. An absorption spectrum was run on each sample, routinely, and the sample was discarded if the color which developed differed from that of glucuronic acid alone.

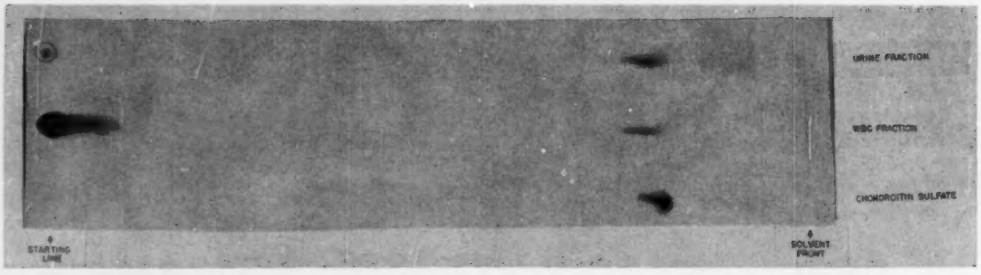

FIG. 2. CHROMATOGRAPHIC COMPARISON OF HUMAN LEUKOCYTE AND URINE FRACTIONS WITH COMMERCIAL CHONDROITIN SULFATE

Solvent 50 per cent ethanol in pH 6.5 M/15 phosphate buffer.

a single spot corresponding to that formed by commercial chondroitin sulfate. The urine fraction, on the other hand, could not be shown to move as a single spot on paper electrophoresis; a streak was formed, the forward portion of which moved at a slightly slower rate than did chondroitin sulfate.[8]

DISCUSSION

Human leukocytes were found to contain appreciable quantities of non-dialyzable material which contained hexuronic acid and hexosamine and which was in final step separated from leukocytes by a method (3) which permitted quantitative recovery of added acid mucopolysaccharides.[9] Chromatographically, the material exhibited a major component which had the same R_f value as the major component of a similar material previously described (1) isolated from human urine. This R_f value was the same, also, as that of the major component of chondroitin sulfate of commercial source. On paper electrophoresis, also, the material from leukocytes formed a spot coinciding with that formed by commercial chondroitin sulfate. The material from leukocytes was considered most probably an acid mu-

[8] Dr. Karl Meyer is very kindly in the process of analyzing the urine fraction at this time. These data will be published separately.

[9] In a control experiment, leukocytes + saline yielded 3.6 gamma of hexuronic acid, saline + chondroitin sulfate, 9.8 gamma, leukocytes + chondroitin sulfate 13.3 gamma. In a control experiment, leukocytes + saline yielded 10.3 gamma of hexosamine, saline + chondroitin sulfate 12.0 gamma, leukocytes + chondroitin sulfate 19.6 gamma. Absorption spectra were run on all samples, as usual.

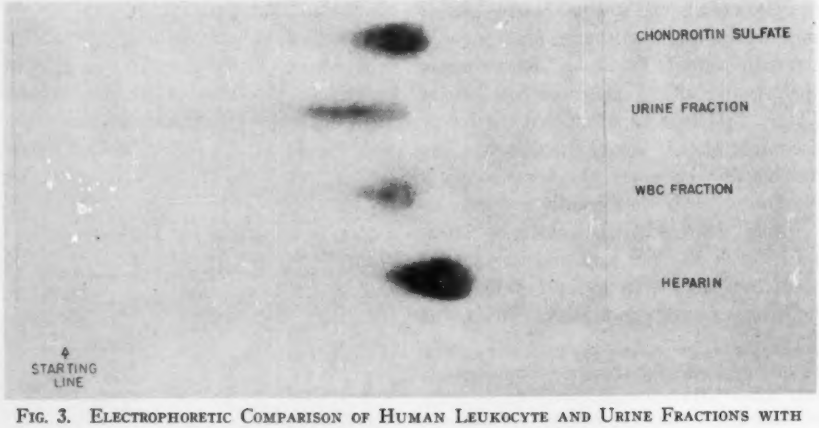

FIG. 3. ELECTROPHORETIC COMPARISON OF HUMAN LEUKOCYTE AND URINE FRACTIONS WITH COMMERCIAL CHONDROITIN SULFATE AND HEPARIN

copolysaccharide on the basis of the findings. Quantitatively, the material pooled from leukocytes of both males and females yielded after dialysis about half the expected carbazole value for hexuronic acid, since equimolar amounts of hexuronic acid and hexosamine are to be expected in most acid mucopolysaccharides. The hexuronic acid content[10] was unchanged by dialysis, whereas appreciable amounts of hexosamine were lost on dialysis.[11] In considering relative amounts of acid mucopolysaccharides in leukocytes, therefore, the hexuronic acid content is the more valid of the two quantitations done on non-dialyzed Astrup fractions (as summarized in Figure 1). The difference in hexuronic acid content of the acid mucopolysaccharides of leukocytes from males and females was small but significant ($p < 0.01$).

The previously described material from urine (1) is similarly regarded as probably being an acid mucopolysaccharide. However, dialysis studies subsequent to the previous report (Table II) have shown that there are present also in the Astrup fraction dialyzable glucuronic acid compounds thus far not identified and not components of acid mucopolysaccharides. These account frequently but not always for the increased Astrup fraction in the urine of diabetics (4). They account also for the bulk of the increase in Astrup fraction following the ingestion of glucuronolactone and for the increased amount of Astrup fraction in the urine of human males compared to females. The

TABLE II

Content of hexosamine and hexuronic acid in Astrup fractions of non-dialyzed and dialyzed 3-hour human urine collections

Urines	In Astrup fraction of 3-hour urine (gamma) (±S.D.)			
	Subjects	Hexuronic acid	Subjects	Hexosamine
Non-dialyzed	11♂	887±186	6♂	1,540±191
Dialyzed	13♂	375±136	13♂	908±292
Non-dialyzed	8♀	557± 83	6♀	1,060±273
Dialyzed	14♀	301± 84	14♀	791±265

remaining non-dialyzable material yielded a comparatively low carbazole value for hexuronic acid. Electrophoretically, on paper, it moved poorly, with formation of a streak rather than a spot, unlike commercial chondroitin sulfate. Further work is in progress to establish the identity of the non-dialyzable, acid mucopolysaccharide-like material. It is to be emphasized that the studies reported here on both leukocytes and urine were done on dialyzed but otherwise unpurified materials.

The relationship of acid mucopolysaccharides of leukocytes to the somewhat similar substance found in urine is conjectural at present. Thomas and Good (12) showed that the bilateral cortical necrosis of the generalized Shwartzman reaction which is associated with the occurrence in glomerular capillaries of Hotchkiss-Schiff staining hyaline material could be prevented in rabbits by first rendering the animals leukopenic by the use of nitrogen mustard. The cortical necrosis was not prevented when the lower extremities were protected by circulatory occlusion from the effects of nitrogen mustard, the extremities later being permitted to contribute normal leukocytes and perhaps other materials to the general circulation. The hyaline material which they observed in the glomerular capillaries of these animals with cortical necrosis was thought to be a mucopolysaccharide because of its histochemical characteristics. It cannot be said from their data whether the material was deposited or originated at the site of occurrence. Mucopolysaccharide similar to that isolated from urine and leukocytes in the present study can be demonstrated readily also in beef kidney tissue (Figure 4) and in all likelihood in kidney tissue of other species. However, in view of the fact that leukocytes (or other body com-

[10] Follette, Valentine, Hardin, and Lawrence (11) used a naphthoresorcinol method for measuring hexuronic acids of leukocytes in contrast to the carbazole method of the present study, so direct comparisons may not be justifield. However, it is of interest that they noted a total glucuronic acid content of 3,655 gamma per 10^{10} leukocytes (mean value) with range of 2,720 to 5,120 gamma. Thus, perhaps about one-third of the total glucuronic acid content of leukocytes can be accounted for in the acid mucopolysaccharide fraction.

[11] The identity of the dialyzable hexosamine component is not known. As noted in Figure 1 (representing yields of hexuronic acid and hexosamine from non-dialyzed Astrup fraction), the quantity of dialyzable hexosamine was probably greater in samples from females than from males. This observation is based on the data presented in Figure 1, assuming that the proportion of hexosamine to hexuronic acid is stable after dialysis and having determined that the hexuronic acid content is unchanged on dialysis. The assumption is reasonable but not validated at present.

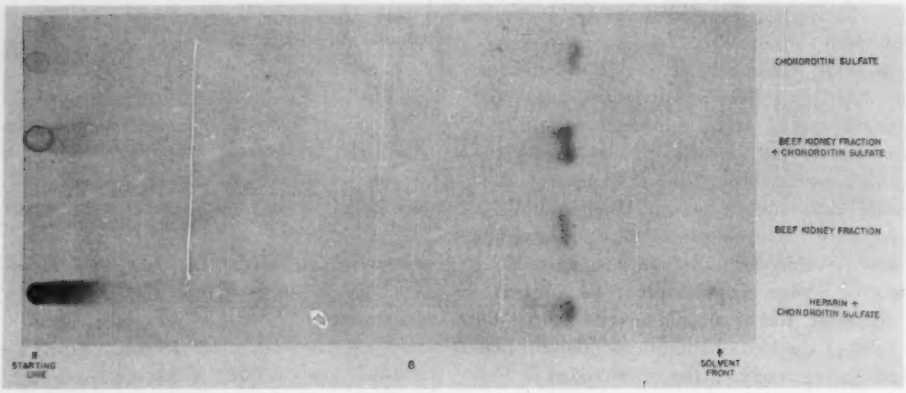

FIG. 4. CHROMATOGRAPHIC COMPARISON OF BEEF KIDNEY FRACTION WITH COMMERCIAL CHONDROITIN SULFATE AND HEPARIN

Solvent 50 per cent ethanol in pH 6.5 M/15 phosphate buffer.

ponents susceptible to elimination or alteration by nitrogen mustard) appear to be essential for the production of the lesion described by Thomas and Good, it is tempting to postulate a connection between the mucopolysaccharides found in leukocytes and the hyaline mucopolysaccharide-containing material observed in the glomerular lesions.

The question of whether the acid mucopolysaccharides of leukocytes contribute to the somewhat similar material found in normal urine remains unanswered. When several rabbits were made leukopenic with nitrogen mustard, their urinary excretion of acid mucopolysaccharides persisted unchanged. However, they were not severely leukopenic at this time, total leukocyte counts being 680 and 1,240 (compared to baseline counts of 7,750 and 6,800) per cmm., respectively, in the only two animals maintaining their previous level of urine output during the period of observation.

It is entirely possible that the acid mucopolysaccharides of leukocytes simply contribute to the structure of the cell. If so, there remains to be investigated whether abnormalities of content or composition of the material in the leukocyte can be demonstrated in disease states and whether such abnormalities would reflect changes going on simultaneously in other less accessible tissues of the body. Preliminary observations in a variety of disease states, including some of the connective tissue diseases, have not as yet revealed consistent quantitative differences.

SUMMARY

1. A non-dialyzable material (or materials) containing hexuronic acid and hexosamine has been separated from human leukocytes and urine by a method which permits quantitative recovery of added acid mucopolysaccharides. The material from both sources yielded a low carbazole value for hexuronic acid as compared to hexosamine yield. Chromatographically the material showed resemblance in its properties to certain components of the inhomogeneous commercial chondroitin sulfate with which it was compared. Electrophoretically on paper, also, the leukocyte material resembled commercial chondroitin sulfate in its migration. The urine material differed, forming a slower streak instead of a spot.

2. All of the hexuronic acid in the fraction separated from leukocytes was associated with the non-dialyzable material. The average yield of hexuronic acid from the fraction representing 10^{10} leukocytes was $1{,}180 \pm 51.6$ (S.E.) gamma in males, 910 ± 47.5 (S.E.) gamma in females. The difference was small but significant ($p < 0.01$).

3. Not all of the hexosamine in the fraction separated from leukocytes was associated with the non-dialyzable material. The dialyzable hexosamine component was not identified.

4. Not all of the hexuronic acid and hexosamine in the fraction separated from urine was associated with the non-dialyzable material. That portion of each lost on dialysis cannot be regarded as be-

ing a component of an acid mucopolysaccharide. After dialysis of the fraction, no difference in quantity of non-dialyzable material was found between males and females.

ACKNOWLEDGMENT

The technical assistance of Mrs. Elizabeth Corcoran and Miss Sarah Hart is gratefully acknowledged.

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AN APPRAISAL OF METHODS OF TISSUE CHLORIDE ANALYSIS: THE TOTAL CARCASS CHLORIDE, EXCHANGEABLE CHLORIDE, POTASSIUM AND WATER OF THE RAT[1]

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Recent evidence (1, 2) promises to establish even more firmly the older concept that chloride in muscle is predominantly extracellular (3–5). On this premise investigators may find further credence in the use of chloride for the study of exchanges of fluid and electrolyte between the extra- and intracellular phases of this tissue. Obviously a method of high accuracy for the determination of tissue chloride is necessary if knowledge concerning body cell composition is to progress. Concern as to the accuracy of present methods arises when one considers the normal values for the chloride content of muscle, as obtained in various laboratories. Perusal of the literature reveals values for normal rat muscle ranging from 3.13 to 6.41 mEq. per 100 grams of fat free, dry solid (6, 7). Although the homogeneity of the tissue samples and/or the methods of determination may be questioned, the argument remains that as yet no means are at hand for ascertaining whether all the chloride in tissue has been measured (8).

Apart from the analyses of isolated tissues, investigations by many workers have sought to determine the extracellular volume and electrolytes in the whole animal by measuring the volume of distribution of various substances that either follow the distribution of chloride or do not penetrate cell water. If these data are to be compared with the volume of distribution of an isotope of chloride or bromide in the whole animal (9), it is important that we know how closely the volume of distribution of Cl^{36}, Cl^{38}, or Br predicts the true total body chloride.

The present investigation attempts to throw some light on these questions by comparing in rats the total body chloride, as determined by the distribution of stable bromide, with the actual total chloride as determined by carcass analysis. Because the success of such a study depends in large part on the exact determination of carcass chloride, the accuracy of existing methods for tissue chloride also had to be assessed. The methods investigated were the commonly used open Carius method, employing silver nitrate-nitric acid digestion, and an alkaline ashing procedure with final determination of chloride titrimetrically, using the Conway microdiffusion technique. The accuracy of the methods have been assessed, first, by comparing the results with those for total body chloride determined by bromide distribution, and secondly, by relating carcass chloride to lean body mass. Total body water and total potassium also have been measured in rats of varying ages and weights and related to lean body mass.

METHODS

Thirty-five rats of varying weights and ages were placed on a low residue diet[2] containing 26 mEq. of sodium and chloride and 167 mEq. of potassium per kilogram. Ten additional rats were placed on the same diet but 0.9 per cent sodium chloride solution was given for drinking in place of tap water. After one week the animals were injected intramuscularly with 2 per cent sodium bromide, 0.3 ml. per 100 grams body weight, from a preweighed syringe, and two to three hours later their

[1] This investigation was supported in part by research grants (H-1638) from the National Heart Institute and (A-306) from the National Institute of Arthritis and Metabolic Diseases of the National Institutes of Health, United States Public Health Service.

[2] The constituents of the diet in grams per kilogram of the final mixture were as follows: vitamin free casein, 300; vitamin fortification mixture (Nutritional Biochemicals Corp., Cleveland, Ohio), 20; lard, 100; sucrose, 270; dextrose, 270; $CaCO_3$, 13.5; $Ca(H_2PO_4)_2$, 1.7; $Ca_3(PO_4)_2$, 1.7; $Fe(C_6H_7O_7)\cdot 3H_2O$, 1.2; $MnSO_4\cdot 4H_2O$, 0.2; $ZnCl_2$, 0.01; $CuSO_4$, 0.01; $MgSO_4$, 4.6; K_2HPO_4, 14.5; KI, 0.07; NaCl, 1.5.

fur was removed by electric clippers under Nembutal® anesthesia. The animals were weighed, and blood was drawn from the aorta for serum bromide and chloride determinations. Each animal was then sacrificed, the carcass cut longitudinally, placed in a weighed beaker and dried to a constant weight at 105° C. over a four-day period. The blood drawn for bromide and chloride determination was centrifuged, the volume of serum measured, and the red cells added to the carcass before drying. Total body water was calculated from the weight loss. When drying was complete, the carcass was crushed to a homogeneous aggregate in a mortar and a 40-gram specimen (representing all, or nearly all, of the carcass in most instances) was placed in four or five Soxhlet thimbles, weighed, and fat extracted for eight hours with petroleum ether in the Soxhlet apparatus. The thimbles were then removed, exposed to the air for two hours, placed in a drying oven at 105° C. for one hour, reweighed, and the amount of fat extracted was calculated from the weight loss. The fat free, dry carcass was then reduced to a 60 mesh powder by grinding in a Wiley laboratory mill and homogenized by thorough mixing. The carcass was then considered to be suitable for analysis and placed in an air-tight container.

The methods for serum bromide determination and for calculating total chloride have been previously described (10, 11). For convenience with respect to ashing of the serum for the bromide determination, the following slight modification was introduced. The one-ml. aliquot of serum was placed in a platinum crucible, four drops of 2 N KOH added, and the mixture digested on a water bath for two hours. When the digestion mixture was dry, the crucible was placed in a muffle furnace at 550° C for one hour. After cooling, the ash was dissolved in 1.2 or 1.5 ml. of 0.3 N H_2SO_4 and a one-ml. aliquot placed in the outer chamber of the No. 2 porcelain Conway unit for bromide determination (12). Bromide assays were done in duplicate on each rat. To obtain a blank value for serum bromide, serum was taken from six additional rats which had been on the same diet and had not received bromide. This value, resulting from traces of bromide in the diet, averaged 20 μgm. of bromide per ml. In rats receiving bromide, the serum bromide concentrations were 160 μgm. per ml. or slightly greater. In the experiments in which saline replaced tap water for drinking, urine was collected after injection of bromide and correction was made for the small amount of bromide excreted prior to exsanguination. In the rats receiving tap water, the amount of bromide excreted during this period was not detectable.

Serum chloride was determined by the method of Van Slyke (13).

For the determination of carcass chloride, three procedures were used: 1) The open Carius method with digestion by heating on a hot plate; 2) the open Carius method with digestion in a boiling water bath; and 3) alkaline ashing of the carcass with chloride determination by the microdiffusion technique of Conway (12).

The procedure for the open Carius method was that of Van Slyke (13) with the modifications of Wilson and Ball (14) and of Hastings and Eichelberger (15). In both methods 1) and 2) above, the procedure was the same except for the temperature of digestion. Samples of powdered carcass weighing approximately 150 mg. were added to 1.0 ml. of 0.075 N $AgNO_3$ and the sides of the tube washed with 5 ml. of water. The mixture was then allowed to stand for 16 to 20 hours at 4° C. Two ml. of concentrated nitric acid were then added and the mixture digested for two to four hours with frank boiling but without bumping on a hot plate at "medium" heat (method 1), or by immersion for 6 hours in a boiling water bath (method 2). During digestion the tubes were covered either with inverted glass thimbles or with funnels. The temperature of the mixture during digestion using the hot plate for heating was 104° to 105° C. and, using the water bath, 96° to 97° C. Several drops of hydrogen peroxide were added after digestion had proceeded for a time. At completion of digestion under these conditions, the mixtures were nearly colorless. After cooling the tubes, ferric alum was added and the mixture titrated with 0.02 N NH_4CNS in an ice bath.

For determination of chloride by the alkaline ash-microdiffusion method, samples of powdered carcass weighing approximately 150 milligrams were placed in platinum crucibles, 0.5 ml. of 2 N KOH were added, and the mixture digested on a steam bath for two hours. When the digestion mixture was dry, the crucible with cover was placed in a muffle furnace at 550° C. for one hour. A white ash was obtained and after cooling, 0.3 ml. of 4 N H_2SO_4 were added and the contents of the crucible washed over into a 10-ml. volumetric flask and made to volume with distilled water. Four aliquots, each of 1.0-ml. volume, were placed in the outer chambers of four No. 2 porcelain Conway units[3] and chloride estimated by liberating free chlorine and displacing an equivalent amount of iodine from potassium iodide as described by Conway (12). Following the two-hour period of microdiffusion, the free iodine liberated was titrated with a microburette containing 0.01 N $Na_2S_2O_3$, using starch as the indicator. With each set of determinations, standards, consisting of 1.0 ml. of a 4 mEq. per liter solution of KCl, were run in quadruplicate. For reasons which were not apparent, the titration figure for the standards varied slightly from day to day. By using the titration factors for standards which had been run concurrently, reproducible values were obtained for the unknowns.

For potassium estimation 150 milligrams of carcass

[3] The following procedure was used in the cleaning of the Conway dishes: The units were first soaked in very dilute oxalic acid solution to remove permanganate stains, washed in running tap water, heated to boiling in a strong solution of a chloride-free detergent, wiped thoroughly with muslin and placed in running tap water. They were then soaked in 0.02 N sulfuric acid, rinsed once in tap water and eight to ten times in distilled water and finally dried in an oven at 105° C. Careful attention to cleaning was essential for reproducibility of the titrations.

TABLE I

Rat no.	Weight gm.	Fat free dry solid gm.	Total water ml.	Lean body mass gm.	Carcass chloride* mEq.	Bromide exch. chloride mEq.	Carcass potassium mEq.
6	237.0	50.0	151.0	201.0	7.07	6.65	
8	231.0	52.2	161.8	214.2	7.98	8.00	
10	220.0	57.6	150.3	208.9	8.23	7.13	14.60
12	200.4	46.9	146.0	192.9	7.13	8.20	13.70
13	183.0	42.6	132.4	175.0	6.57	6.40	
14	223.3	51.3	162.8	214.1	8.49	8.30	14.76
15	228.0	54.0	152.2	206.2	7.85	7.60	14.03
16	158.0	38.7	117.5	156.2	6.22	5.94	12.42
17	131.0	33.3	96.7	130.0	5.82	5.45	
18	435.0	104.3	308.3	412.6	15.45	14.10	
19	303.5	73.6	217.0	290.6	9.73	8.80	20.63
20	184.6	42.7	139.0	181.7	7.45	6.62	11.93
21	395.5	91.9	276.5	368.4	13.51	12.40	
22	346.0	84.0	239.0	323.0	11.52	12.30	23.80
23	207.5	46.1	135.5	181.6	7.19	7.75	12.68
24	254.5	69.2	175.8	245.0	9.11	8.85	17.74
25	294.0	69.8	191.8	261.6	9.87		18.55
26	233.2	54.3	160.2	214.5	8.30	9.75	15.60
27	380.0		257.0			11.82	
28	332.0	71.8	214.0	285.8	10.70	10.40	22.0
29	333.5		205.0			10.55	
30	341.5	73.0	206.3	279.5	10.90	11.60	21.65
31	296.0	66.4	197.8	264.2	9.65	11.04	19.15
32	281.0	59.4	174.6	234.0	8.93	8.90	17.30
33	231.1	50.1	150.2	200.3	7.70	8.30	15.00
34	354.2	95.2	216.0	311.2	11.99	11.40	
35	286.0	61.9	182.3	244.2	9.40	9.72	17.50
36	74.5	16.3	55.5	71.8	2.96	3.00	5.12
37	82.5	18.4	59.3	77.7	3.43		5.67
38	74.0	15.7	55.1	70.8	3.15	3.35	5.45
39	268.0	60.6	181.0	241.6	8.95	9.13	17.70
40	78.0	16.0	57.4	23.4	3.40	3.96	5.35
41	92.5	20.6	66.4	87.0	3.41		5.96
42	235.0	54.5	152.2	206.7	7.70	7.92	15.85
43	237	53.9	155.7	209.6	7.95	7.92	15.40
44	293.5	69.0	192.5	261.5	10.30	10.92	20.60
45	211.0	47.9	139.5	187.4	7.50	7.15	13.80
46	294.8	63.0	180.7	243.7	9.28	9.24	18.50
			Rats receiving isotonic saline				
1	259.0	62.9	177.7	240.6	8.81	9.84	
2	248.7	60.4	170.3	230.7	9.14	8.52	
3	267.6	66.1	182.6	248.7	9.88	9.42	
4	247.5	60.3	166.6	226.9	8.54	9.00	
5	280.0	69.2	192.2	261.4	9.90	10.20	
48	260.2	60.4	170.5	230.9	9.29	9.28	
49	307.0	74.6	204.5	279.1	10.97	10.65	
51	281.5	66.8	195.0	261.8	9.55	9.94	
52	261.5	59.5	166.0	225.5	9.04	9.40	
53	269.0	66.8	179.5	246.3	8.95	9.15	

* Determined by alkaline ash-microdiffusion method. Corrected for chloride content of serum taken for analysis

were weighed into a platinum crucible and ashed at 550° C. for 12 hours. The ash was taken up with 1 ml. of 1 N HCl, washed over into a 50-ml. volumetric flask and made up to volume. Aliquots of this solution were used for the flame photometer, for potassium analyses.

RESULTS

The agreement between replicate determinations (Tables I and II) of carcass chloride by each of the three methods has been assessed by calculation of the standard error of analysis [4] from the results of

[4] The standard error of analysis gives the range within one standard deviation in which the results of analysis will fall about a theoretical parameter expressing the average of an infinite number of similar analyses. The range for the average of duplicate analyses is obtained by dividing the SE_a for a single analysis by $\sqrt{2}$; for the average of triplicate analyses, by dividing by $\sqrt{3}$, *etc.*

TABLE II

*Standard error of analysis (SE_a) for three methods of carcass chloride determination**

Method	No. of Duplicates	Average Cl Content	SE_a For Single Determination	SE_a For Av. of Duplicates
Open Carius Hot Plate	53	13.46	± .335	± .237
Open Carius Water Bath	55	14.51	± .488	± .345
Alkaline Ash-Microdiffusion	50	14.14	± .189	± .134

* Values in mEq. per 100 gm. fat free, dry, solid.

TABLE III

*Recovery of chloride added to carcass**

Chloride In Carcass Sample	Chloride Added	Chloride Obtained	Chloride Expected	Per Cent Recovery
		Open Carius - Hot Plate		
2.14	2.0	4.23	4.14	104
1.88	2.0	4.00	3.88	106
1.82	2.0	4.22	3.82	117
1.61	2.0	3.66	3.61	102
Means		4.03	3.86	
		Open Carius - Water Bath		
2.46	2.0	4.48	4.46	101
1.86	2.0	3.96	3.86	105
2.48	2.0	4.57	4.48	104
2.08	2.0	4.24	4.08	108
Means		4.31	4.22	
		Alkaline Ash - Microdiffusion		
2.22	1.0	3.21	3.22	99
2.22	1.0	3.23	3.22	101
2.20	1.0	3.22	3.20	102
2.17	1.0	3.14	3.17	97
2.41	1.0	3.44	3.41	103
2.25	1.0	3.24	3.25	99
Means		3.25	3.25	

* Values are in μEq.

TABLE IV

Results of carcass chloride analysis by open Carius and alkaline ash-microdiffusion methods †

Carcass No.	A Alkaline Ash	B Open Carius Hot Plate	C Per Cent Difference $\frac{B-A}{A} \times 100$	D Open Carius Water Bath	E Per Cent Difference $\frac{D-A}{A} \times 100$
6	13.70	13.07 (8)	-4.6	13.99	+2.1
8	14.84	14.12 (8)	-5.2	15.51	+4.2
10	13.97	12.84	-8.1 *	14.14 (6)	+1.2
12	14.77	14.12	-4.3	14.77	0
13	14.95	12.81 (6)	-14.3*	15.07	+1.0
14	15.49	13.72 (6)	-11.4 *	15.37	-0.8
15	13.97	12.51 (5)	-10.5 *	14.33	+2.6
16	15.28	13.59	-11.1 *	15.57	+2.6
17	16.65	14.72	- 9.6 *	16.25	-2.4
18	14.32	13.58	- 4.9 *	14.19	-0.9
19	12.85	12.53	- 2.4	12.57 (7)	-2.2
20	17.10	16.53	- 3.3	16.61 (8)	-2.9
21	14.17	13.89	- 1.3	14.19 (6)	0
22	13.48	12.38	- 8.2 *	14.40 (6)	+6.8 *
23	14.93	13.82	- 7.4 *	14.59	-2.3
24	12.87	12.76	- 0.8	14.32	+11.3 *
25	13.99	13.90 (6)	- 0.1	14.61	+4.4
26	14.63	13.11 (8)	-10.3 *	14.34	-2.0
28	14.20	11.97 (6)	-15.7 *	13.55	-4.6 *
30	14.40	11.61	-19.4 *	13.12	-8.9 *
31	13.87	14.80	+ 6.7 *	13.77	-0.7
32	14.33	14.62	+ 2.0	13.23	-7.7
Means			- 7.0		+0.42

*Differences significant at 5 per cent level or better (t test (16)).

Figures in parenthesis indicate the number of determinations averaged. For values which are not marked, the determinations were either in triplicate (alkaline ash method) or in quadruplicate (open Carius methods).

† Values in mEq. per 100 gm. fat free, dry solid.

duplicate determinations. The formula used was

$$SE_a = \sqrt{\frac{\Sigma d^2}{2N}}$$

in which SE_a is the standard error of analysis, N, the number of paired observations, and d, the difference between the results of duplicate analyses. The standard errors, calculated from approximately 50 duplicate analyses by each of the three methods, are shown in Table II. Reproducibility was best for determinations by the alkaline ash-microdiffusion method [5] and poorest with the open Carius method with water bath digestion.

The results of recovery experiments with each

[5] A single analysis by the alkaline ash-microdiffusion method was taken as the result of a single ashing. Since the chloride content of each ash was determined by quadruplicate analyses by the microdiffusion procedure, the titrimetric error for this method is negligible as compared with the open Carius method in which a single titration determined the result of each analysis.

TABLE V

Results of muscle chloride analysis by open Carius and alkaline ash-microdiffusion method *

Alkaline Ash	Open Carius Hot Plate	Open Carius Water Bath
Human Muscle (Autopsy)		
13.44	--	12.66
18.22	--	17.94
9.54	--	8.77
8.78	--	6.85
10.42	--	10.12
9.97	--	10.27
16.36	--	14.88
22.27	--	19.47
Dog Muscle		
7.33	7.36	6.70
Rat Muscle		
6.15	3.14	5.50

* Values in mEq. per 100 gm. fat free, dry solid.

of the three methods are given in Table III. Chloride was added to fat free, dry carcass as potassium chloride. With the open Carius techniques, the amount of chloride recovered tended to be greater than the amount added. Agreement between expected and found was much better with the alkaline ash-microdiffusion method.

Table IV compares the values for carcass chloride as determined by each of the three methods. The values represent the means of triplicate analyses by the alkaline ash-microdiffusion method and of quadruplicate or more analyses by the Carius methods. For the comparison, the values obtained by the alkaline ash method have been used as the standard of reference. With the open Carius procedure and hot plate digestion, the values were usually lower than those obtained with the alkaline ash-microdiffusion method.[6] The mean percentage difference was −7.0. In over half of the instances, the differences were statistically significant ($p < .05$). On the other hand, with digestion on a boiling water bath, the results using the open Carius method were, in the main, comparable to those obtained by the alkaline ash method. In only four of the 22 instances were the Carius values significantly lower ($p < .05$) than those obtained by the alkaline ash procedure. The average difference amounted to + 0.4 per cent.

In Table V values for muscle analyses for chloride by each of the three methods are compared. The specimens of muscle were obtained from children who had died from various diseases and from a normal dog and rat. In the majority of instances, the results for the Carius method employing a water bath are slightly lower than the values obtained by alkaline ashing.

In Figure 1 the total chloride as determined from the volume of distribution of bromide has been plotted against total chloride determined by carcass analysis by the alkaline ash-microdiffusion technique. The data are for 35 rats receiving tap water (solid circles) and for 10 rats receiving 0.9 per cent NaCl for drinking (open circles). For both groups the points fall fairly close to the

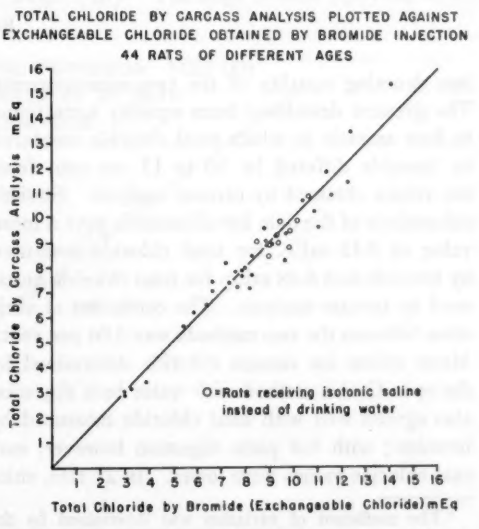
TOTAL CHLORIDE BY CARCASS ANALYSIS PLOTTED AGAINST EXCHANGEABLE CHLORIDE OBTAINED BY BROMIDE INJECTION
44 RATS OF DIFFERENT AGES
Total Chloride by Carcass Analysis mEq
Total Chloride by Bromide (Exchangeable Chloride) mEq
O=Rats receiving isotonic saline instead of drinking water

FIG. 1. TOTAL CHLORIDE BY CARCASS ANALYSIS (ALKALINE ASH-MICRODIFFUSION METHOD) VS. BROMIDE EXCHANGEABLE CHLORIDE

The line denotes equality of the two measurements.

[6] In reconciling the generally lower values for carcass chloride obtained by the hot plate-open Carius method with the fact that recovery experiments with this method usually yielded too much chloride, it can only be assumed that the original analyses of the specimens to which chloride was added were in error. If these values were too low, and the analyses after chloride addition more nearly correct, the apparent recovery of too great an amount of chloride would result.

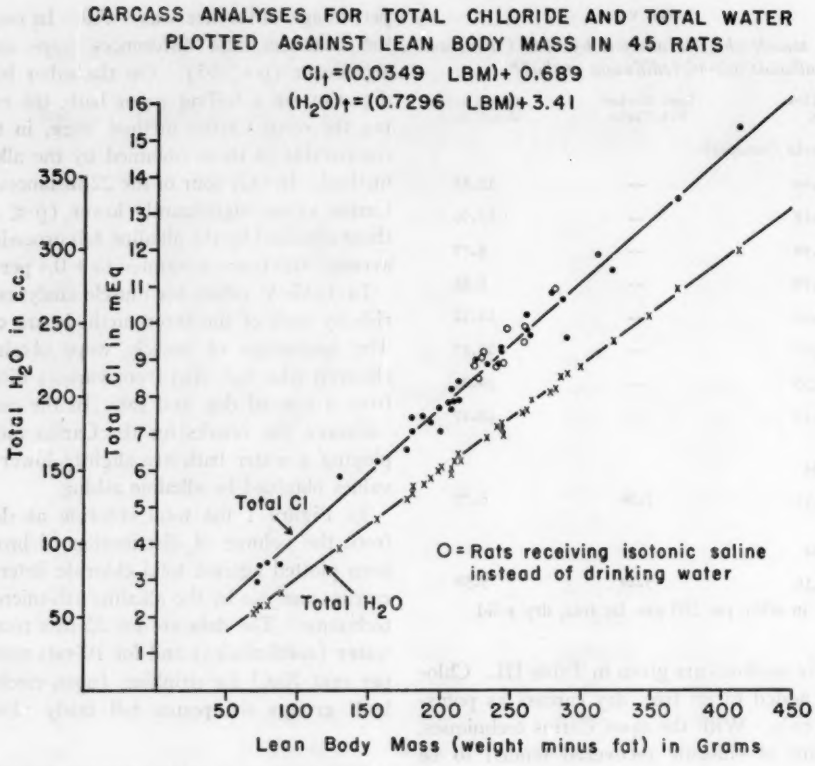
CARCASS ANALYSES FOR TOTAL CHLORIDE AND TOTAL WATER
PLOTTED AGAINST LEAN BODY MASS IN 45 RATS
Cl_t=(0.0349 LBM)+ 0.689
$(H_2O)_t$=(0.7296 LBM)+3.41
Total H_2O in c.c.
Total Cl in mEq
Total Cl
Total H_2O
O= Rats receiving isotonic saline instead of drinking water
Lean Body Mass (weight minus fat) in Grams

FIGURE 2

line denoting equality of the two measurements. The greatest deviations from equality were found in four animals in which total chloride measured by bromide differed by 10 to 15 per cent from the values obtained by carcass analysis. Statistical analysis of the data for all animals gave a mean value of 8.45 mEq. for total chloride measured by bromide and 8.48 mEq. for total chloride measured by carcass analysis. The coefficient of variation between the two methods was 5.04 per cent.[7] Mean values for carcass chloride determined by the open Carius method with water bath digestion also agreed well with total chloride measured by bromide; with hot plate digestion however, carcass chloride values were lower. In 21 rats, chloride measured by bromide distribution averaged 8.87 mEq. as compared with 8.91 mEq. by carcass analysis by the open Carius-water bath digestion method, and 8.36 mEq. by the open Carius method with hot plate digestion. Judging from the results of the alkaline ash and open Carius-water bath digestion methods, the exchangeable chloride as measured by bromide would seem to be very close to true total body chloride. Failure of agreement between exchangeable chloride and carcass chloride as seen in certain animals may be attributable to the summation of technical errors, particularly in the exchangeable chloride method. It should be recalled that the determination of total chloride indirectly by bromide distribution depends on accurate measurements of the amount of bromide injected, of the serum bromide and chloride levels, and assumes the blank value for serum bromide to be the same in all rats. The carcass analysis, on the other hand,

[7] The coefficient of variation was determined by the expression, $SE_a/\text{Mean} \times 100$, in which SE_a is the standard error of analysis calculated as described in the text from the results of the alkaline ash and exchangeable chloride estimations for each rat.

depends only on accuracy in the measurement of the weight of fat free, dry solid and tissue chloride.

In Figure 2, carcass chloride, determined by the alkaline ash-microdiffusion method, and total body water, obtained by desiccation, have been plotted against lean body mass (weight minus ether-extractable fat). Over the range investigated, both total water and total chloride are linearly related to lean body mass, not only in the rats maintained on tap water but also in those given 0.9 per cent NaCl for drinking. The regression equations expressing the relations between these variables, calculated by the method of least squares, were

$$Cl_t = 0.0349 \text{ LBM} + 0.689 \qquad (1)$$

and

$$(H_2O)_t = 0.730 \text{ LBM} + 3.41 \qquad (2)$$

in which Cl_t is the total chloride in mEq., $(H_2O)_t$, total water in ml. and LBM, the lean body mass in grams. The standard error of the estimate for the regression of total chloride *vs.* LBM was ± 0.38 mEq. and for that of total water *vs.* LBM, ± 2.1 ml.

Since LBM is, by definition, the sum of the fat free, dry solids and total water (FFDS + $(H_2O)_t$), the expression LBM − FFDS may be substituted for $(H_2O)_t$ in equation (*2*) and LBM expressed in terms of fat free, dry solid as

$$\text{LBM} = 3.69 \text{ FFDS} + 12.61$$

When carcass chloride determined by the open Carius method with water bath digestion was plotted *vs.* lean body mass, the configuration was almost identical to that shown in Figure 2 for the alkaline ash method. The regression, calculated for 22 rats, was also similar to equation (*1*);

$$Cl_t = 0.0338 \text{ LBM} + 0.910$$

The standard error of the estimate was somewhat greater, amounting to ± 0.44 mEq.

The relation between total body potassium (K_t), as obtained by carcass analysis, and lean body mass is shown in Figure 3. Again, a linear relation pertains. The calculated regression was

$$K_t = 0.0747 \text{ LBM} - 0.330$$

The standard error of the estimate was ± 0.70 mEq.

The equation for total water *vs.* lean body mass (equation *2*) is similar to that calculated by Pace and Rathbun (17) from the data of Ashworth and Cowgill (18). The slope in their equation, 0.721,

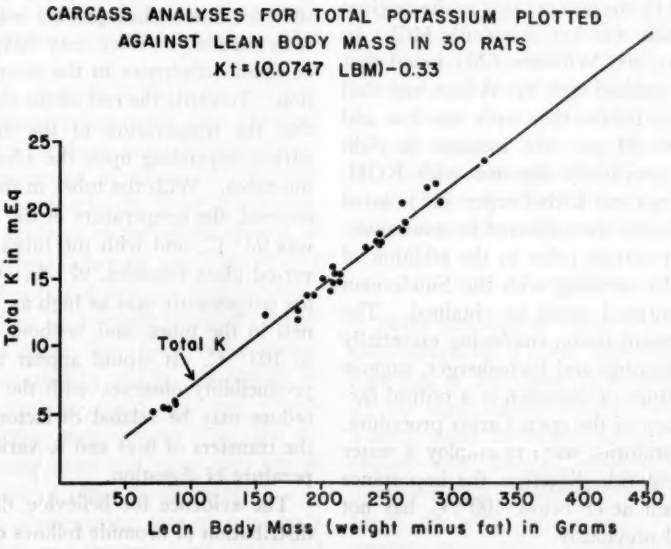

FIGURE 3

is almost identical, while the intercept, 1.76 is lower, probably because smaller rats were used. For the adult rats in the present series, the body water expressed as percentage of lean body mass is 73.8, a value in good agreement with the average value of 73.2 derived by Pace and Rathbun (17) from their own work and that of others on a number of species.

It should be noted that the intercepts in the equations given above indicate that the young rat will have a greater chloride and water content and a somewhat lower potassium content per unit of lean body mass than the adult. Data by Iob and Swanson (19) indicate that in the newborn rat, the relationships between electrolyte content and weight may be curvilinear. For rats two to three weeks of age, their data agree well with those of the present study.

DISCUSSION

The open Carius method for the determination of chloride in biological fluids was originally introduced by Van Slyke in 1923 (13). In 1928, Wilson and Ball (14) proposed that the method be modified by adding silver nitrate and nitric acid separately, rather than combined in a single reagent as originally described. By adding the silver nitrate first, higher values were obtained. It was suggested by the authors that in the original procedure, chloride was lost as volatile HCl. In 1933, Sunderman and Williams (20) found that the open Carius method with the Wilson and Ball modification gave results that were too low and obtained a 10 to 20 per cent increase in yield if tissues were previously digested with KOH. However, Hastings and Eichelberger (15) stated that if fat free tissues were allowed to stand overnight with silver nitrate prior to the addition of nitric acid, results agreeing with the Sunderman and Williams' method could be obtained. The results of the present study, employing essentially the method of Hastings and Eichelberger, suggest that the temperature of digestion is a critical factor in the accuracy of the open Carius procedure. While most laboratories seem to employ a water bath for tissue chloride digestion, the importance of controlling heat at or below 100° C. has not been emphasized previously.

In our hands, the alkaline ash-microdiffusion method was superior to the open Carius procedure. Not only were replicate determinations in better agreement and recovery of added chloride more satisfactory, but also the values agreed well with the indirect determination of chloride by bromide distribution. Furthermore, carcass chloride, determined by this procedure, showed little scatter when plotted against lean body mass. It should be noted that with the alkaline ash method, titrimetric error can be reduced to a minimum since multiple titrations can be done on a single ashing. Because the ash of replicate specimens agreed well in chloride content, a single ashing may be done with little sacrifice of accuracy and considerable economy of tissue.

The source of error with the open Carius method is somewhat obscure. The lower values generally obtained when the hot plate was used suggest that chloride may be lost during digestion at high temperatures. Supporting this assumption is the fact that digestion on a boiling water bath usually gave higher values which were in agreement with the alkaline ash procedure. On the other hand, there were indications that other factors may be operative. It was frequently noted that whereas multiple analyses run concurrently often showed good agreement, another series of analyses on the same specimen at a later time gave values differing significantly. The factor responsible for these discrepancies was not determined with certainty. They may have been the result of small differences in the temperature of digestion. Towards the end of the study, it was found that the temperature of the digestion mixtures varied, depending upon the adequacy of covering the tubes. With the tubes in the water bath, uncovered, the temperature of the digestion mixture was 93° C., and with the tubes covered with inverted glass thimbles, 97° C. On the hot plate, the temperature was as high as 105° C. with funnels in the tubes, and, without the funnels, 102° to 103° C. It would appear that the poor reproducibility observed with the open Carius procedure may be related to factors concerned with the transfers of heat and to variations in the temperature of digestion.

The evidence for believing that the volume of distribution of bromide follows closely the volume of distribution of chloride is substantial (21–26).

TABLE VI

Investigators	Method	Number of dogs	mEq. Cl/Kg.	Remarks
Harrison, Darrow, and Yannet (5)	Carcass analysis	2	33.3	Gastrointestinal juices washed out and discarded
Winkler, Elkinton, and Eisenman (28)	Cl^{38}	3	32.2	Six-hour space
Cheek and West (29)	Stable Br	13	32.5	Three-hour space
Gamble, Robertson, Hannigan, Foster, and Farr (24)	Br^{82}	4	32.5	Three-hour space
	Cl^{36}	4	30.6	Three-hour space
	Br^{82}	4	35.2	24-hour space
	Cl^{36}	4	32.5	24-hour space
Burch, Threefoot, and Ray (30)	Cl^{36}	5	42.5	Measured over period of days; losses in feces and urine estimated; young dogs used
Swan, Madisso, and Pitts (9)	Cl^{36}	5	35.4	Lean dogs used Six-hour space
Weir (26)	Stable Br	5	35.5	24-hour space Cl = 39.5 mEq./kilo of lean weight

The volumes of distribution of Cl^{36}, Cl^{38} and Br^{82} agree closely when determined simultaneously in man (24) or dog (25). The present finding that the mean values for exchangeable chloride approximate closely the carcass chloride under normal conditions, and conditions of salt loading, lends further credence to the above supposition. The data would indicate that in the rat, as noted by others in the dog (27) and human (10, 24), a three-hour period for equilibration of bromide seems to be sufficient for penetration of the chloride space.

Fat is a variable quantity in the body and it is not possible to compare accurately data obtained from one animal with those from another unless the results are expressed in terms of lean body mass or fat free, dry solid. In Table VI the results of chloride analyses of the whole carcass of two dogs by Harrison, Darrow, and Yannet (5) may be compared with the data for dogs obtained by isotopes of Cl or by bromide distribution. It can be seen that these data even when uncorrected for fat, compare favorably. It should be noted in passing that the results of Burch, Threefoot, and Ray (30) were derived from the investigation of young dogs and their data are at variance with those of other investigators.

Many workers in presenting data on total body electrolyte have followed the principles established by Needham (31) and have plotted total electrolyte on a double logarithmic grid using body weight as a standard of reference (11, 19, 32, 33). Such plots are of value in that they emphasize the exponential nature of growth. It has been shown that from early life to maturity, chemical growth with respect to chloride and sodium can be expressed by a simple exponential function (11, 32). When data for total electrolyte (34) are plotted against body weight on an arithmetic grid, considerable scatter is evident whereas in double logarithmic plots the scatter is not conspicuous. It would appear that a large part of such scatter is the result of the variable contribution of body fat to body weight. The present study demonstrates that for chloride and potassium in the rat, a close correlation exists between the body content of these electrolytes and lean body mass.

SUMMARY

Three methods of tissue chloride analysis have been compared: 1) The open Carius method with digestion of the tissue by boiling on a hot plate; 2) the open Carius method with digestion in a boiling water bath; and 3) an alkaline ash procedure with determination of chloride by the microdiffusion technique of Conway. Of the three methods, the alkaline ash-microdiffusion method gave better agreement with respect to

repeated analyses. When chloride was added to fat free, dry carcass, the microdiffusion method demonstrated a more satisfactory recovery. Although the values obtained by the open Carius method with boiling water bath digestion were less reproducible than with the alkaline ash method, the averages of replicate determinations by the two methods were in good agreement. On the other hand, the employment of a hot plate for digestion during the Carius procedure frequently gave values that were significantly low. The heat at which digestion is carried out appears to be an important factor in the accuracy of the open Carius procedure.

The exchangeable chloride has been measured in 45 rats by determination of the volume of distribution of bromide. The same rats were then sacrificed and reduced to a fat-free, dry, fine, homogeneous powder suitable for chloride analysis. It was found that total carcass chloride in rats closely approximates exchangeable chloride. The substitution of isotonic saline for drinking water did not alter exchangeable chloride or carcass chloride to any appreciable extent.

The agreement obtained for total rat chloride by the use of bromide and by two methods of carcass analysis supports the contention that all the chloride in the rat has been accurately accounted for.

It has been found that total body chloride, water and potassium exhibit a simple linear relationship to lean body mass. Such relationships should facilitate the investigation of abnormal states of electrolyte balance.

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THERMAL BURNS IN MAN. IX. URINARY AMINO ACID PATTERNS[1]

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It is a well known phenomenon that burned patients lose considerable amounts of nitrogenous materials following the initial trauma. They continue to stay in negative nitrogen balance for several days or even a month or more despite efforts to supply quantities of the necessary amino acids for metabolism and repair. Even though much protein material is lost in exudates (1), considerable amounts of catabolic nitrogenous substances appear in the urine. Besides the urea, creatinine, uric acid, and ammonia, relatively large amounts of amino acids and amino acid derivatives are excreted. This increase in the loss of amino acids is of special interest since it represents, for the most part, a wastage of materials needed for replacement and repair of the injured tissue.

In a recent paper Nardi (2) has studied the excretion of the free amino acids in urine of the burned patient by qualitative paper chromatographic techniques. In the study reported here, the quantitative determination of the amounts of 16 amino acids excreted in the urine following burn trauma has been studied in 9 patients to define with more exactness the qualitative and the quantitative aspects of the amino acid loss. In order to determine with greater clarity the amino acid patterns in urines of these burned patients, the amount of "free" amino acid and the "total" amount of each amino acid were determined. The amount of each amino acid in the "bound" form was determined by the difference between the two values found.

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METHODS

Patients with burns involving more than 15 per cent of the body surface were examined in the emergency ward by the burn study team and then admitted to the metabolic ward where initial clinical measurements were made. The patients were then placed on a program of fluid therapy approximating that suggested by Evans, Purnell, Robinett, Batchelor, and Martin (3). This consistent policy was required to permit comparison among patients. The details of studies on these patients, other than the amino acid excretion studies, appear elsewhere. Description of each patient included in this report has already been published (4).

As soon as was feasible, usually about 48 hours following admission to the hospital, the patient was placed on a standardized liquid oral diet. It was of known content both as to quality and quantity of the various components. Amino acids were supplied in the diet as whole natural protein. This standard diet procedure was used in order to eliminate any metabolic variations in the excretion of amino acids that might occur from variations in the quantity or quality of the amino acid source ingested.

The patient was continued on the diet for at least four days. Complete urine collections were made and an aliquot of the collection on the fourth day of the diet was analyzed for the amino acid content. During late convalescence from the burn, usually just prior to discharge from the hospital, the diet was again administered to five of the nine patients for four days and urines again collected. On several occasions the patients were readmitted to the hospital for these latter studies. Thus in a measure the patient served as his own control for estimation of the change in the amino acid excretion brought about by the trauma of the burn.

The methods of handling the urines and performing the microbiological assays for the amino acids are those described by Eades and Pollack (5). In general, the method involved the microbiological assay of 16 amino acids using a uniform medium and one microorganism, *Leuconostoc mesenteroides* P-60 ATCC No. 8042. Amino acids in the urine were determined quantitatively both in "free" and "total" amounts. The "free" amount of each acid was assayed in a urease treated aliquot of the original urine while the "total" amount was determined in an acid hydrolyzed aliquot of the original urine. Cystine and tryptophan were not assayed in the hydrolyzed samples as there was destruction of these two in the hydrolysis procedures.

TABLE I

Amino acids excreted by burned patients (mg./24 hrs.)

Patient	J. H.		T. S.		L. O.		M. L.		G. S.	
% Burn	55%		17%		22%		18%		35%	
Days Post-burn	6		6		6		6		6	
Amino Acid	Free	Total	Free	Total	Free	Total	Free	Total	Free	Total
Glycine	602	803	480	850	278	578	345	755	482	862
L. Glutamic Acid	0–T.*	1,026	0–T.	640	0–T.	779	0–T.	580	0–T.	896
L. Aspartic Acid	0–T.	613	50.4	390	40.3	182	177	645	23.8	1,030
L. Leucine	19.0	234	27.0	130	26.8	126	41.3	178	38.5	420
L. Valine	8.0	201	35.3	212	20.3	123	62.7	156	36.2	455
L. Phenylalanine	50.9	234	97.0	258	43.4	52.5	39.7	186	28.0	158
L. Threonine	0–T.	195	85.0	140	98.0	133	80.0	210	0–T.	331
L. Lysine	25.0	178	61.6	196	74.9	213	158	236	43.2	399
L. Isoleucine	0–T.	78.1	20.4	75	19.8	96.3	7.3	189	45.0	319
L. Arginine	0–T.	335	42.0	80	0–T.	55.6	0–T.	146	0–T.	508
L. Histidine	134	185	148	188	230	250	390	395	211	438
L. Proline	112	491	73.0	180	42.8	152	18.6	131	23.0	456
L. Tyrosine	50.2	223	156	260	78.0	227	159	221	66.7	301
L. Methionine	29.3	112	22.0	90.1	19.4	32.5	15.7	37.6	10.3	99.6
L. Tryptophan	40.1	—	58.0	—	53.5	—	95.0	—	68.6	—
L. Cystine	244	—	126	—	168	—	198	—	220	—

TABLE I—*Continued*

Amino acids excreted by burned patients (mg./24 hrs.)

Patient	A. Ni.		O. H.		M. R.		R. J.	
% Burn	40%		25%		23%		55%	
Days Post-burn	8		7		5		11	
Amino Acid	Free	Total	Free	Total	Free	Total	Free	Total
Glycine	181	1,126	432	950	445	730	203	1,119
L. Glutamic Acid	46.2	399	0–T.	752	0–T.	240	0–T.	670
L. Aspartic Acid	77.2	266	60.0	792	49.8	220	34.7	159
L. Leucine	3.6	92.4	15.0	190	5.8	94.6	15.4	286
L. Valine	3.4	115	28.3	242	0–T.	94.7	0–T.	285
L. Phenylalanine	24.5	460	56.3	255	18.6	89.6	35.0	187
L. Threonine	0–T.	124	143	420	0–T.	50.0	56.0	452
L. Lysine	33.2	133	50.6	321	43.9	74.4	26.1	410
L. Isoleucine	0–T.	110	37.5	236	0–T.	52.7	16.3	332
L. Arginine	0–T.	155	16.2	152	0–T.	57.5	24.2	212
L. Histidine	158	218	318	680	91.3	156	129	185
L. Proline	0–T.	159	125	330	87.8	129	23.9	466
L. Tyrosine	60.9	125	138	188	14.9	62.5	65.3	231
L. Methionine	11.3	40.0	23.5	48.0	25.4	60.8	37.3	119
L. Tryptophan	69	—	67.5	—	12.2	—	57.8	—
L. Cystine	54.1	—	270	—	223	—	224	—

* 0–T.—Zero to trace quantities by microbiological assay.

RESULTS

The amounts of the 16 amnio acids excreted in 24 hours by nine burned patients are shown in Table I in both "free" and "total" [5] amounts. In Table II, values for the amino acids excreted by five of these patients late in convalescence are shown. For comparison the mean normal values, with the standard error, for 10 normal subjects (5) are included to show that upon recovery from the burn trauma, the amino acid values approach normalcy again. Statistical evaluation of the data on the burned, as compared to the recovered patient, was made using the "student's" t Method (6). The probability level arbitrarily considered significant in this evaluation was that the difference between means could occur by chance alone less than 5 per cent of the time ($P < 0.05$). Of the 14 amino acids evaluated (excluding tryptophan and cystine) only 4, aspartic acid, isoleucine, proline, and valine, gave P values greater than 0.05. With the exception of isoleucine even these amino acids had P values between 0.05 and 0.1. For most of the amino acids the P values were between 0.02 and 0.05 with tyrosine giving a value < 0.001.

[5] Except cystine and tryptophan.

TABLE II

Amino acids excreted by convalescent burned patients (mg./24 hrs.)

Patient	J. H.		T. S.		L. O.		M. L.		G. S.			
% Burn	55%		17%		22%		18%		35%		Mean normal values (3) mg./24 hrs.	
Days Post-burn	95		85		24		62		80			
Amino Acid	Free	Total	Free	Total	Free	Total	Free	Total	Free	Total	Free*	Total*
Glycine	199	353	296	400	219	395	118	314	145	503	226±15	405±42
L. Glutamic Acid	0–T.	161	0–T.	232	0–T.	132	0–T.	288	0–T.	586	1± 1	170±27
L. Aspartic Acid	31.5	176	0–T.	269	30.3	201	26.5	191	52	200	12± 2	161±13
L. Leucine	14.0	45.6	13.0	87.5	5.5	29.4	8.0	29.5	16.5	57	9± 1	33± 2
L. Valine	0–T.	100	19.4	110	0–T.	40.1	7.9	33.0	18.9	30.5	9± 3	35± 5
L. Phenylalanine	4.5	24.8	19.4	73.2	14.7	28.9	19.0	32.4	13.3	37.0	15± 3	25± 3
L. Threonine	0–T.	44.9	24.1	90.0	0–T.	88.0	12	27.0	0–T.	73.0	19± 4	40± 4
L. Lysine	12.5	46.4	25.0	115	0–T.	72.1	26.8	65.6	75.6	155	18± 3	52± 6
L. Isoleucine	15.2	41.2	32.2	90.7	20.6	67.7	22.8	36.4	22.5	33.6	4± 1	24± 3
L. Arginine	0–T.	52.8	0–T.	69.5	0–T.	50.9	0–T.	43.7	0–T.	184	2± 1	22± 3
L. Histidine	40.0	60.8	139	149	120	145	132	136	25.6	46.4	125±11	171±27
L. Proline	10.9	57.6	11.0	111	22.2	99.7	9.3	59.0	8.8	97.0	6± 1	33± 3
L. Tyrosine	9.3	42.1	54.4	90.0	53.4	92.5	26.0	70.4	22.4	70.1	27± 3	48± 6
L. Methionine	13.9	25.6	0–T.	20.0	18.0	31.8	4.2	16.4	3.0	30	9± 3	26± 7
L. Tryptophan	12.0	—	25.0	—	13.4	—	19.4	—	9.3	—	—	—
L. Cystine	71.2	—	79.4	—	69.4	—	76.0	—	29	—	—	—

* ± Value is the standard error of mean.

At the 5 per cent statistical level no significant difference between the amount of the various amino acids excreted by the normal control and the recovered burn patient could be shown with the possible exception of aspartic acid, isoleucine, and proline. It should be kept in mind, however, that statistical evaluation of so few data is not quite reassuring and with a few more data these differences might not be significant.

A comparison of the data of the controls with the data of the burned patients yielded results comparable to the comparison of the burned with the recovered patients.

DISCUSSION

From the tables it can be seen that all 16 of the amino acids assayed were excreted in higher amounts by the 5 patients in the immediate post-burn period than were excreted by the same patients later in convalescence. For all 9 patients the same holds true when comparison is made to the excretion values of the 10 normal subjects. The amounts of the bound forms of the amino acids appearing in the urine immediately following the burn are considerable but decrease in quantity as recovery progresses. The amount of bound histidine, however, is not great in either the early or late samples. Three other amino acids, threonine, isoleucine, and methionine, are variable in the amounts of bound forms excreted by the various patients but they are somewhat lower than most of the amino acids. Protein was not detected in the urines that were assayed for the amino acids; therefore the bound forms would appear to represent peptides, conjugates, or other forms of amino acid derivatives. This "bound form" aspect of the amino-aciduria is of considerable interest (7, 8), as it may yield information on intermediary metabolism and metabolic pathways of amino acid utilization.

Most striking is the amount of bound glutamic acid that is lost during the immediate post-burn period and the absence, or nearly so, of the free glutamic acid in all but one of the samples, burn or normal, that were assayed. Glutamine is present in the urine and may account for some of the bound form. The amount of bound aspartic acid is also considerable in the urine of the recently burned patient, but the value returns to normal with recovery. Another most interesting observation is that glycine is excreted in relatively large amounts, free and bound, immediately post-burn. This increase follows closely the rise of many other metabolites found in the urine. Of particular interest is the parallelism of the adrenocortical response to the burn trauma and the increase of the excretion of amino acids. The amounts of the corticoids excreted are usually high immediately after the burn and return to a normal value upon recovery (4). This increase in urinary corticoids may in part account for some of the bound glycine excreted (8) the gly-

cine being bound in some fashion to the steroid. Especially does this seem plausible since it has been reported (9) that the steroid content of urine increases following enzymatic or acid hydrolysis. This treatment also gives rise to more free amino acids.

Of all 16 amino acids that were quantitatively assayed, relatively few were found to be absent, or in trace amounts, in the free state and none was absent when the hydrolyzed samples were tested. Even though results are not reported in this paper on the excretion values for alanine or serine, due to the variable results obtained in our assay, the findings in our laboratory are that alanine and serine both are excreted in appreciable amounts. Our consistent microbiological assay results showing the presence of free tyrosine, tryptophan, and phenylalanine in every case are in contrast somewhat to the report of Nardi (2) who showed free tyrosine in only one case. Of the fourteen urines assayed, free arginine was low or absent in eleven, free valine in four and free threonine in five. Free aspartic acid was found in all but two of the fourteen samples. Whether the aspartic acid was excreted in the free state or in some relatively labile bound form has not been definitely determined.

Our results tend to support the idea that the severity of the burn influences the degree of the amino acid loss in the urine. Especially does this seem to be evident in the amount of the bound forms excreted. The increase in the amino acids in the urine may be due to an increased demand for amino acids for the repair of the injured tissues, possibly controlled by some mechanism mediated through the pituitary-adrenal system, as an increase in the corticoids accompanies this phenomenon. Since it has been demonstrated that an increase in amino acid output follows the injection of ACTH (10), it is reasonable to assume that trauma from burns will cause a concomitant rise in ACTH and consequently an increase in aminoaciduria. These facts implicate the general body reaction to stress as being, in part at least, responsible for the often detected increase in amino acid loss in many traumatic conditions be they mild or severe. Whether this alarm response affects the excretion mechanism of the kidney or simply raises the level of the plasma amino acids above the functioning threshold has not been settled.

SUMMARY

The excretion patterns of 16 amino acids have been determined in the urine of patients immediately post-burn and later in convalescence. The amounts of free and bound forms of the amino acids are greater in the immediate post-burn period than in the later period of convalescence. Large amounts of glutamic acid are found in the bound form with none or only a trace appearing in the free form in both the burned and normal patient's urine. Likewise glycine is excreted in large amounts, free and combined, immediately post-burn. The values return to normal upon recovery. Of all 16 amino acids that were quantitatively assayed relatively few were absent as free amino acids and none were absent when the hydrolyzed urines were tested. Our results for the most part tend to support the idea that the severity of the burn bears a close relationship to the degree of the amino acid loss in the urine.

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THE RATES OF SYNTHESIS AND THE TRANSPORT OF PLASMA FATTY ACID FRACTIONS IN MAN [1]

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Comparatively little is known regarding the metabolism of fatty acids in the plasma of man. The present investigation was undertaken in order to study the formation of fatty acids from acetate-1-C^{14} and the relative rates at which these substances form esters with the various plasma lipid fractions under normal circumstances. In addition, an attempt was made to test *in vivo* the concept that fatty acids and cholesterol compete for available "acetate" which is an essential building block for both substances (1). This portion of the investigation was performed under conditions whereby the utilization of acetate for the biosynthesis of cholesterol was considerably diminished by diluting the higher precursor pool through prefeeding with large quantities of squalene (2).

MATERIALS AND METHODS

Seven patients with limited life expectancies were selected for study. All were considered to be in a good nutritional state and clinically free from metabolic disturbances. Pertinent data on these patients are listed in Table I.

The subjects were maintained on normal hospital diets during the course of the investigation. Two hundred microcuries of acetate-1-C^{14} (specific activity 1.0 mc per mM) were dissolved in a convenient volume of tap water and administered orally to all patients in the postabsorptive state on the morning of the experiment. Three patients, N. D., J. G., and J. R., were fed 25 grams of "natural squalene" [3] per day in divided doses for three days prior to and two days after the administration of the radioactive compound. Serial blood samples were taken in heparinized syringes beginning one hour after the administration of acetate and continued at intervals for 48 hours. The isolation of the plasma C^{14} fatty acid fractions was carried out by a slight modification of the methods described by Popják and Beeckmans (3) and Pihl and Bloch (4). The material designated as the fatty acids of the neutral fat of the plasma by the latter observers are those lipids which are soluble in acetone. These include the fatty acids from triglycerides, from cholesterol esters, and the free fatty acids. Since the term "neutral fat" properly refers to the triglycerides alone the expression "non-phospholipid" fatty acids will be applied to the combination of lipids which are found in the supernatant acetone. The phospholipid fatty acids, on the other hand, are those fractions precipitated from a petroleum ether solution by acetone and $MgCl_2$.

Ten to twenty cc. of plasma was usually extracted with 250 to 500 cc. of 3:1 ethanol-ether and filtered. The precipitate was washed with an additional 50 to 100 cc. of the ethanol-ether mixture; the filtrate was then concentrated to 1 to 4 cc. *in vacuo* under nitrogen and the residue was extracted four times with 20 to 25 cc. portions of warmed petroleum ether.

During tests on the specificity of the method it was found that the volume to which the alcohol-ether extract was concentrated was a critical factor in obtaining adequate recoveries (90 to 100 per cent). By analyzing the lipid phosphorus content of the original filtrate and comparing it with the petroleum ether extract the following observations were made. If the quantity of residue remaining after concentration amounted to 5 cc. or more, only 50 to 75 per cent of the lipid phosphorus could be recovered in the petroleum ether system despite repeated vigorous extractions. If the residue was taken down just to dryness, complete removal from the walls of the flask was difficult, only 50 to 80 per cent of the lipid phosphorus being recovered after long periods of gentle reflexing. Since the partition coefficients of the various phospholipid fractions under these circumstances are not known, accurate control of the volume of the residue (1 to 4 cc. with 90 to 100 per cent recovery) was considered necessary to prevent fractionation and assure extraction of a representative sample of the phospholipid with petroleum ether.

After the extraction of the residue with the solvent the material was concentrated to a small volume and the phospholipid and "non-phospholipid" fatty acids were then separated by the method prescribed by Popják and Beeckmans (3). In this procedure the separation of the two fractions by repeated precipitation with five volumes of acetone and approximately 20 to 30 drops of $MgCl_2$ in ethanol was considered complete since no detectable lipid phosphorus remained in the supernatant acetone.

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[3] Obtained from Distillation Products Inc., Rochester, New York.

TABLE I

Clinical and laboratory data

Patient	Age	Sex	Weight *kilograms*	Diagnosis	Plasma total fatty acids *mEq./L.*		Plasma lipid phosphorus *mg. %*	
Controls								
H. F.	35	F	60	Carcinoma of breast	16.5		9.6	
J. M.	62	M	58	Carcinoma of tonsil	12.9		8.2	
J. L.	37	M	78.5	Oligodendroglioma	10.9		7.2	
J. R.	47	M	70	Carcinoma of lung	8.5		5.8	
N. R.	59	M	61	Carcinoma of stomach	10.3		6.8	
					Before Squalene	After	Before Squalene	After
Squalene-fed								
N. D.	25	M	55.5	Functioning carcinoma of adrenal—removed one month prior to study	10.2	12.2	6.4	7.8
J. G.	63	M	62.7	Carcinoma of colon	11.0	10.1	6.1	7.0
J. R.*	47	M	70	Carcinoma of lung	9.4	10.2	6.6	7.9

* Patient J. R. was repeated as his own control eight weeks after first study.

The phospholipids were dissolved in 10 to 20 cc. of moist ether and saponified by the addition of an equal volume of 80 per cent ethanol and 40 per cent (w/v) aqueous KOH at 70 to 80 degrees for 24 hours. As the saponification progressed, more and more water was added and at the end all the organic solvent was evaporated off. The soaps were transferred with hot water to separatory funnels, made acid with H_2SO_4 and the fatty acids extracted with petroleum ether.

The acetone supernatants obtained from the phospholipid precipitations were evaporated to dryness by distillation and the residues saponified with ethanolic KOH at 80 degrees for six hours. The unsaponifiable material was then removed by extraction with petroleum

TABLE II

*Specific activity of plasma fatty acid fractions**

Control subjects

Hours	J. R. NPFA†	P‡	H. F. NPFA	P	J. M. NPFA	P	N. R. NPFA	P	J. L. NPFA	P
½							166	21	158	14
1	331		405		1,190	8	428	67	940	42
2	1,085		858	27	1,312	14	537	69	1,327	41
4	731	139	357	58	943	78	477	117	1,159	107
8	468	287	303	107	740	288	254	264	630	291
10							103	181	591	331
11	395	310	231	174	369	425				
12							155	252	406	431
14	257	417	185	277	596	500	184	331	455	373
24	156	200	153	183	175	334	99	239	282	310
48	110	98	47	46	136	150	81	93	271	237

Squalene-fed subjects

Hours	N. D. NPFA	P	J. R. NPFA	P	J. G. NPFA	P
1	1,565	92	461	60	380	63
2	1,700	100	851	81	606	102
4	1,385	382	398	127	470	161
8	864	573	309	218	194	278
11	618	758	284	294	234	
14	777	759	256	368	230	362
24	373	530	269	304	215	415
48	205	323	165	222	144	129

* Specific activity = disintegrations per minute per mg. plasma fatty acid fraction.
† Non-phospholipid fatty acids.
‡ Phospholipid fatty acids.

ether. After five extractions the solvents were evaporated off from the soaps on a water bath. The free fatty acids were obtained as described for phospholipids.

In the final step, the washed and purified fatty acids were taken up in small volumes of ether, mounted directly on lens paper in aluminum cups, 4.36 cm.^{2} and weighed, after the solvent was blown off, to an accuracy of 0.1 mg., according to the procedure described by Entenman, Lerner, Chaikoff, and Dauben (5).

Since the saponification and extraction procedure for the determination of the C^{14} content of the purified fatty acid fractions resulted in some loss of material, the total fatty acids and lipid phosphorus of plasma were determined by chemical methods previously described from this laboratory (6–11). The "non-phospholipid" fatty acids were estimated by the formula: "non-phospholipid" fatty acids = total fatty acids − phospholipid fatty acids. Assuming that the average molecular weight of fatty acid to be 269, the fatty acids of phospholipid and "non-phospholipid" fractions in mEq. per liter are converted to milligrams per cent by multiplying by 26.9.

RESULTS

"Non-phospholipid" fatty acids

In all of the subjects studied, the specific activity of the "non-phospholipid" fatty acids of the plasma reached a maximum value at two hours (Table II). It can be assumed on the basis of animal (12) and human (13) experiments that all available acetate-1-C^{14} had been completely utilized and the peak activity of expired $C^{14}O_2$ had already occurred by this time. At this point, the per cent incorporation of C^{14} into the "non-phospholipid" fatty acids of the total circulating plasma ranged from 0.57 to 1.91 for the control group and from 0.80 to 1.68 for the squalene-fed subjects (Table III). With the disappearance of the precursor, the synthesis of tagged fatty acids ceased and the specific activity curves began to fall rapidly during the next six to twelve hours (Figure 1). This phase probably represents the distribution of the recently formed C^{14} fatty acids and their utilization by the body tissues with subsequent dilution and replacement by newly synthesized non-labeled fatty acids. In five of the eight patients, a distinct secondary peak was noted 11 to 14 hours after the administration of radioactive acetate. This may be due to one of the heterogeneous components of the "non-phospholipid" fraction. Following the appearance of this peak, the specific activities of these fractions were usually lower than those of the fatty acids of phospholipids at 24 and 48 hours.

The total fatty acid and lipid phosphorus content of the plasma of all the subjects was within normal limits and was not significantly altered by the administration of squalene (Table I). The small increments noted in the lipid phosphorus after squalene feeding remain unexplained.

Phospholipid fatty acids

The specific activity of the phospholipid fatty acids was low during the first two hours of sampling (Figure 1, Table II). It continued to rise slowly and intersected the specific activity curve of the "non-phospholipid" fatty acid fraction in

TABLE III

Per cent incorporation of C^{14} into plasma fatty acid fractions

Patient	Total fatty acids of the non-phospholipid fraction in circulating plasma (mg.)	DPM*/mg. NPFA† at 2 hours	Per cent incorporation of C^{14} in NPFA at 2 hours	Total P‡ in circulating plasma at 14 hours (mg.)	DPM/mg. P at 14 hours	Per cent incorporation of C^{14} in P at 14 hours
Controls						
H. F.	7,911	858	1.52	4,066	277	0.255
J. M.	5,886	1,312	1.75	3,380	500	0.384
J. L.	6,354	1,327	1.91	3,991	373	0.338
J. R.	4,316	1,085	1.07	2,878	417	0.272
N. R.	4,713	537	0.57	2,885	331	0.217
Squalene-fed						
N. D.	4,375	1,700	1.68	2,467	759	0.425
J. G.	5,696	606	0.80	2,657	415	0.250
J. R.	4,756	851	0.91	3,219	368	0.269

* DPM = Disintegrations per minute.
† NPFA = Non-phospholipid fatty acids.
‡ P = Phospholipid fatty acids.

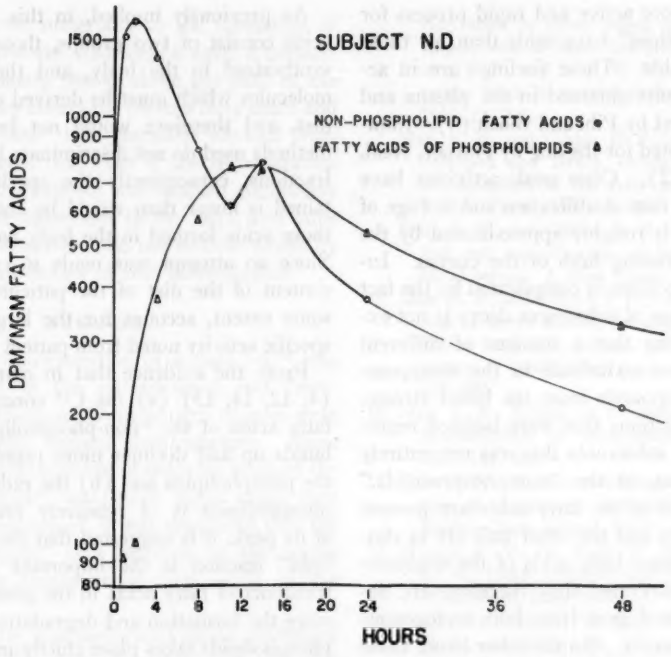

FIG. 1. THE INCORPORATION OF ACETATE-1-C^{14} INTO THE FATTY ACIDS OF PHOSPHOLIPIDS AND "NON-PHOSPHOLIPIDS"

about 8 to 14 hours. In all the subjects except one, J. L., the specific activity reached a maximum sometime between 14 and 24 hours, and then declined at a rate at times comparable to that of the "non-phospholipid" fatty acids. At the peak (14 hours) the per cent C^{14} incorporated into the fatty acids of phospholipids was essentially the same for both groups of subjects, ranging from 0.217 per cent to 0.384 per cent for the controls and from 0.250 per cent to 0.425 per cent for the squalene-fed patients (Table III).

DISCUSSION

The main source of energy in the body is probably derived from the breakdown of fatty acids. In the form of triglycerides these substances may be stored in fat depots and represent a readily available fuel supply. Since a large portion of utilizable carbohydrate is converted to fat prior to oxidation and an appreciable quantity of the triglyceride found in the body is derived from exogenous sources, the transportation of large quantities of fatty acids to and from the fat depots, presumably by way of the plasma, must occur during the metabolism of these foodstuffs. Previously, it had been assumed that the phospholipids of the plasma played a major role in the transfer of fatty acids from the liver to the tissues. However, on the basis of recent animal studies (4, 14, 15) it would now appear that the "non-phospholipids" function as the important vehicle in this respect.

The dynamics involved in the synthesis of the plasma lipids of man are shown in Figure 1. The ascending phase of the specific activity curves for the phospholipid and "non-phospholipid" fraction is a reflection of several processes. These include the rates of formation of fatty acids from acetate, probably chiefly in the liver, the relative rates of incorporation of fatty acids into the phospholipid and "non-phospholipid" molecule, and the subsequent secretion of these substances into the plasma. The height and position of the peak of the curve in time probably represents the culmination of these events. From a comparative analysis of the curves it appears that the formation of ester link-

ages is a much more active and rapid process for the "non-phospholipid" fatty acids than for those of the phospholipids. These findings are in accord with the results obtained in the plasma and liver slices of the rat by Pihl and Bloch (4). Similar results were noted for the dog by Harper, Neal, and Hlavacek (12). Once peak activities have been attained, the rate of utilization and storage of these compounds is roughly approximated by the slope of the descending limb of the curves. Interpretation of this slope is complicated by the fact that for both groups of substances decay is not exponential, indicating that a number of different metabolic processes contribute to the disappearance of these compounds from the blood stream. Since the lipid fractions that were isolated represent a mixture of substances this was not entirely unexpected. Thus, in the "non-phospholipids," approximately half of the fatty acids are present as the triglycerides and the other half are in cholesterol esters. Since fatty acids of the triglycerides are usually saturated they therefore are derived to a variable degree from both endogenous and exogenous sources. On the other hand, those associated with cholesterol are mostly "essential" fatty acids and are supplied by the diet. The presence of these highly unsaturated fatty acids in the "non-phospholipid" fraction would tend to dilute and lower the carbon-14 content since they are not synthesized from short chain carbon compounds to any appreciable degree by the mammalian organism.

The turnover of the "non-phospholipid" fatty acids appears to be very prompt. This, to a large extent, is probably due to the rapid exchange of fatty acids between the liver and depots via the plasma (12). In addition to this dilution by non-labeled fatty acids, some peripheral utilization occurs. The build up and decline in the radioactivity of the fatty acids of the plasma phospholipids however is much slower. Apparently on the basis of animal investigation (12) this fraction is rapidly formed in the liver reaching peak activity in about a half an hour. However, it is only slowly released into the plasma. This observation differs from that of Zilversmit, Entenman, and Chaikoff (16) who by using P-32 noted a continuous reutilization of the tag into the phospholipid molecule over a period of several hours.

As previously implied, in this study the fatty acids consist of two groups, those which can be synthesized in the body, and those unsaturated molecules which must be derived chiefly from the diet, and therefore would not be labeled. The methods used do not discriminate between the two fractions, consequently the specific activity obtained is lower than would be anticipated if only those acids formed in the body had been studied. Since no attempt was made to control the lipid content of the diet of the patients, this could to some extent, account for the large variations in specific activity noted from patient to patient.

From the evidence that in man as in animals (4, 12, 14, 15) (a) the C^{14} concentration in the fatty acids of the "non-phospholipids" of plasma builds up and declines more rapidly than that in the phospholipids and (b) the radioactivity of the phospholipids is of relatively low concentration at its peak, it is suggested that the "non-phospholipid" fraction is the important vehicle for the transport of fatty acids in the plasma. However, since the formation and degradation of the plasma phospholipids takes place chiefly in the liver (15), these substances may also function to a limited extent as carriers of fatty acids from this organ to the tissues.

In a previous communication (2) evidence was presented to show that the hydrocarbon squalene may be an intermediate in the synthesis of plasma cholesterol in man. When labeled acetate was given to human subjects prefed with squalene, the specific activity of the plasma cholesterol, although highly variable, was much less than that noted in the controls. This effect was thought to be due primarily to a dilution of the "intermediate pool" between acetate and cholesterol by the exogenous hydrocarbon. However, in this present investigation, when the plasma fatty acids of these patients were isolated and their radioactivity determined, the isotope concentrations in these fractions, although also variable, were essentially the same in the control and squalene-fed subjects. Squalene is unique among known cholesterol precursors since it alone enters the cholesterol molecule without previously breaking down to two carbon fragments (17–20).

From the results obtained, the acetate normally used for cholesterol synthesis when large quantities of squalene are administered does not ap-

pear to contribute materially to the formation of fatty acids. However, following a dose of acetate-1-C^{14} approximately two to three per cent of the total radioactivity enters the plasma fatty acids and cholesterol of man. It is therefore obvious from the data presented that even if the incorporation of acetate into cholesterol was blocked completely by squalene, the increment in the fatty acid label that may have occurred could not be determined by the methods and design of this investigation.

SUMMARY

The rates of incorporation of acetate-1-C^{14} into the fatty acids of the phospholipid and "non-phospholipid" fractions of the plasma of man were determined under normal circumstances. Since the formation and turnover of the fatty acids of the "non-phospholipids" was much more rapid than that of the phospholipid, it was suggested that the "non-phospholipids" may function as the major vehicle for the transport of fatty acids in the plasma.

A similar study was performed under conditions whereby the utilization of acetate for the biosynthesis of cholesterol was significantly decreased by prefeeding with large quantities of squalene. This failed to alter the incorporation of acetate into the fatty acids. The results of this phase of the investigation are discussed.

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STUDIES ON COPPER METABOLISM. XVI. RADIOACTIVE COPPER STUDIES IN NORMAL SUBJECTS AND IN PATIENTS WITH HEPATOLENTICULAR DEGENERATION [1]

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A number of abnormalities in the metabolism of copper in hepatolenticular degeneration (Wilson's disease) have now been described. These may be enumerated briefly as follows: the total plasma copper level is usually decreased (1–3); there is a decrease in the concentration of ceruloplasmin (3, 4), the blue copper containing alpha globulin of plasma (5); the direct-reacting fraction of plasma copper (6), that which is bound probably to albumin (7) is increased (2, 7); the urinary excretion of copper is greatly increased (1, 2); the concentration of copper in the tissues is elevated (2, 8); and the patients retain a greater amount of dietary copper than do normal subjects (2, 9, 10).

The pathogenesis of this disease is poorly understood. It has been suggested by several observers (1, 2) that the excessive accumulation of copper in the brain and liver causes the characteristic lesions in these organs. Whether the excessive deposition of copper is due to increased absorption or to a decreased rate of excretion has not been clarified by non-isotopic copper balance studies (2). The observation of Matthews (10) that a larger proportion of an orally administered dose of radioactive copper was recovered in the feces of control subjects than in those of patients with Wilson's disease does not answer this question, because it is not possible to determine by such a study whether more radiocopper was absorbed or whether less had been re-excreted.

The purpose of this paper is to present studies on the excretion of radiocopper in the urine and stools of normal subjects, patients with Wilson's disease, and patients with alcoholic cirrhosis of the liver following the oral and intravenous administration of radiocopper. In addition, the uptake of the isotope into plasma, erythrocytes, and liver will be reported. The amount of radioactivity in the direct-reacting fraction (albumin-bound) of plasma copper and in the indirect-reacting fraction (globulin-bound or ceruloplasmin) was also studied. For this purpose plasma was fractionated in some instances with rabbit anti-human ceruloplasmin serum, and in other cases with ammonium sulfate, because of limitations in the amount of available anti-serum.

Several preliminary reports of this work have been published (11, 12). While the present paper was in preparation, Bearn and Kunkel (13) have reported that the fecal excretion of intravenously administered radiocopper was significantly less in two patients with Wilson's disease than in two control subjects.

METHODS

Eleven normal subjects, four patients with hepatolenticular degeneration, and two patients with alcoholic cirrhosis of the liver were used in these studies. All individuals were hospitalized in the metabolic ward. The normal subjects were healthy males, 25 to 30 years of age. A complete physical examination, urine analysis and determination of volume of packed red cells, leukocyte count, sedimentation rate, and total plasma copper were carried out on each. The four patients (B. S., D. C., J. Si., and Dar. H.) with Wilson's disease have been described previously (2, 3). Blood copper values in these four patients are presented in Table I.

Radioactive copper (Cu^{64}) was supplied as a solution of cupric acetate by Abbott Laboratories on allocation from the United States Atomic Energy Commission. The standard solutions, blood, plasma, urine, and stool

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† Dr. Bush died October 6, 1955.

specimens were counted in a well-type, thallium activated sodium iodide crystal, scintillation counter with an efficiency of 5 per cent for Cu^{64}. Two standard solutions were prepared: one a 1:1,000 and the other a 1:100,000 dilution of the stock solution. The syringe from which the material was delivered to the patient was used for the preparation of the concentrated standard. Since the half life of Cu^{64} is 12.8 hours, a record was kept of the exact time at which each count was performed in order to correct the observed activity for radioactive decay. The dilute standard was used to correct the counts for the first 48 hours, and thereafter the concentrated standard was used. The standards were counted twice a day. The counts of the standards were plotted against time on semi-logarithmic paper as "counts per minute injected." The observed counts per minute obtained on samples from the subjects were corrected for decay.

In order to make the data more comparable, each subject or patient was given a dose of one mg. of copper. Since the specific activity of the material at the time of administration varied from 0.8 to 1.2 mc. per mg. of copper, the final data were adjusted to CPM/one mc. of copper administered.

The stools collected during the first 72 hours were pooled, weighed, and thoroughly mixed in a Waring blendor. Triplicate aliquots of each stool were counted. After 72 hours, single stool specimens were weighed, mixed, and counted in triplicate. Stool collections were continued until less than one per cent of the injected dose of Cu^{64} appeared in a single stool (72 to 120 hours).

Twenty-four-hour urine specimens were collected for three consecutive days after the administration of the isotope. During the first 24 hours one-ml. aliquots were taken for counting. After the first 24 hours the urine was concentrated 10:1 or 20:1 and digested by boiling with concentrated sulfuric, nitric, and perchloric acids (14). A one-ml. aliquot of the digest was counted.

The activity in one ml. of whole plasma was determined 0.5, 1, 2, 3, 4, 5, 6, 8, 12, 24, 36, and 48 hours after the administration of copper by the oral route and 10 minutes, 0.5, 1, 2, 4, 6, 12, 24, 36, and 48 hours after the intravenous administration of radiocopper. In most instances an additional aliquot of plasma was fractionated with either rabbit anti-human ceruloplasmin serum (3) or ammonium sulfate or both.

Plasma fractionation with rabbit anti-human ceruloplasmin serum was carried out as follows. One ml. of plasma was added to a sample of rabbit anti-ceruloplasmin serum calculated to contain excess antibody. After incubation at 37° C. for one hour, the mixture was centrifuged in the cold,[3] and the precipitate was washed successively with 3.0 ml. and 2.5 ml. of cold 0.85 per cent saline. It was then dissolved in 1.0 ml. of 0.1 N sodium hydroxide solution and counted. The washings were added to the supernate and the activity in the supernate ("non-ceruloplasmin" copper) was measured. The supernates from the precipitin reactions were tested for excess antibody in order to be certain that all ceruloplasmin had been precipitated.

Plasma was fractionated with ammonium sulfate by a modification of the method of Earl, Moulton, and Selverstone (15). One ml. of a saturated solution of ammonium sulfate was added to an equal volume of plasma. After standing for 15 minutes at room temperature, the mixture was centrifuged at 8,000 rpm for 15 minutes in a Custom Scientific Instrument Angle Head Centrifuge. The supernatant solution was decanted and the precipitate washed with 2 ml. of 50 per cent saturated ammonium sulfate solution. After centrifugation the precipitate was dissolved in 0.1 ml. of concentrated ammonium hydroxide, made up to one ml. with water, and the activity determined. The washing was added to the origi-

TABLE I

Plasma copper and ceruloplasmin values in four patients with hepatolenticular degeneration

Patient	Total plasma copper μg./100 ml.	Direct-reacting copper μg./100 ml.	Indirect-reacting copper μg./100 ml.	Ceruloplasmin mg./100 ml.
B. S.	57	50	7	2
J. Si.	50	37	13	4
Dar. H.	53	34	19	8
D. C.	86	26	60	19
Normal	108±9	5±6	103±11	34±4

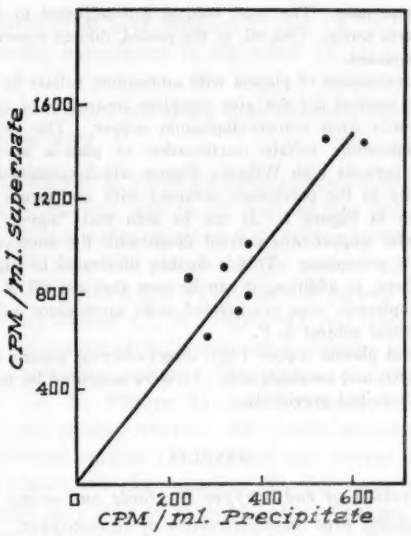

FIG. 1. CORRELATION BETWEEN RADIOACTIVITY OF SUPERNATE AND PRECIPITATE AFTER AMMONIUM SULFATE (50 PER CENT SATURATION) FRACTIONATION OF PLASMA CONTAINING NO DEMONSTRABLE ACTIVITY IN CERULOPLASMIN

The values on the ordinate and abscissa refer to CPM/mc. of radiocopper injected, in the supernate or precipitate obtained from one ml. of plasma.

[3] International PR-1 refrigerated centrifuge.

nal supernate. The total volume was adjusted to four ml. with water. One ml. of the pooled, diluted supernate was counted.

Fractionation of plasma with ammonium sulfate by the above method did not give complete separation of ceruloplasmin from non-ceruloplasmin copper. The results of ammonium sulfate fractionation of plasma samples from patients with Wilson's disease which contained no activity in the precipitate obtained with anti-serum are shown in Figure 1. It can be seen that "non-ceruloplasmin" copper was carried down with the ammonium sulfate precipitate. This is further illustrated in Figure 4, where, in addition, it can be seen that not all of the ceruloplasmin was precipitated with ammonium sulfate in normal subject S. F.

Total plasma copper (16), direct-reacting plasma copper (6), and ceruloplasmin (3) were measured by methods described previously.

RESULTS

Excretion of radiocopper in stools and urine following oral administration of radiocopper

One mg. of radiocopper was administered orally to each of four normal subjects and four patients with Wilson's disease (Table II). The normal subjects excreted 0.1 per cent of the administered activity in the urine; whereas, the patients with hepatolenticular degeneration excreted 14 to 44 times this amount. With the exception of one patient (J. Si.), a significantly smaller amount of radiocopper was recovered in the stools of the patients with Wilson's disease than in the control subjects. Despite the greater amount of activity in the urine of the patients, the total amount of radiocopper recovered was greater in the normal subjects than in the patients, again with the exception of patient J. Si.

TABLE II

Radioactivity in urine and stools following the oral administration of radiocopper

Group	Patient	Per cent of administered activity		
		Urine	Stool	Total
Normal subjects	J. C.	0.1	67.0	67.1
	D. L.	0.1	66.2	66.3
	S. F.	0.1	94.6	94.7
	R. H.	0.1	61.8	61.9
	Mean	0.1	72.4	72.5
Hepatolenticular degeneration	B. S.	4.4	48.8	53.2
	D. C.	1.8	35.5	37.3
	J. Si.	2.2	82.0	84.2
	Dar. H.	1.4	41.9	43.3
	Mean	2.5	52.0	54.5

TABLE III

The influence of potassium sulfide given orally on the radioactivity in the stool, urine, and plasma, following the oral administration of radiocopper

	Patient	No sulfide	With sulfide
Stool	B. S.	48.8	73.8
Per cent of administered activity	D. C.	35.5	110.4
Urine	B. S.	4.4	1.2
Per cent of administered activity	D. C.	1.8	2.1
Plasma maximum *CPM/mc./ml.*	B. S.	2,270	1,730
	D. C.	1,310	472

In order to study the influence of potassium sulfide on the absorption of copper (2), two patients with hepatolenticular degeneration were given one mg. of radioactive copper orally. The activity in the stools, urine, and plasma was measured. Two weeks later, the same two patients were given the same amount of labelled oral copper simultaneously with 20 mg. of potassium sulfide [4] in a gelatin capsule. On both occasions the patients were in a fasting state.

In both patients the per cent of administered activity recovered in the stools increased with administration of potassium sulfide (Table III). In one patient (D. C.), obviously as a result of technical error, the recovery in the stools exceeded 100 per cent. The activity in the urine was less in one patient (B. S.) when sulfide was administered; whereas in the second patient there was no decrease in the activity in the urine. In both patients the maximal activity in the plasma, expressed as CPM/mc. of radiocopper injected per ml. of plasma, was less when sulfide was given than without it.

Excretion of radiocopper in stools and urine following intravenous administration of radiocopper

One mg. of radiocopper was administered intravenously to each of four normal subjects, three patients with Wilson's disease, and two patients with alcoholic cirrhosis of the liver (Table IV). The patients with Wilson's disease excreted

[4] Potash sulfurated technical (Mallinckrodt).

more of the radioactivity in the urine and much less in the stools than the normal subjects. The quantity of radioactive material excreted in the stools of the patients with cirrhosis of the liver was not significantly different from the quantity excreted by the normal subjects. Slightly more activity was recovered in the urine of the patients with cirrhosis than in the urine of control subjects. The total excretion of radiocopper by the normal subjects and the patients with alcoholic cirrhosis of the liver was somewhat greater than by the patients with hepatolenticular degeneration, with the exception of patient J. Si. who excreted a total of 11.2 per cent of the administered activity due to the large percentage (7.8) of the administered dose which was recovered in the urine.

There was no correlation between the degree of impairment of liver function, determined by routine liver function tests, and the amount of radiocopper which was recovered in the stools (Tables IV and V). The amount of radioactive material which was recovered in the stools of the three patients with Wilson's disease was reduced about equally, even though there was marked impairment of liver function in patient D. C.; whereas, in B. S. and J. Si. there was no demonstrable abnormality in liver function, either clinically or by liver function tests. Furthermore, the two patients with alcoholic cirrhosis of the liver excreted more than 3 times as much of the administered radiocopper as the patients with Wilson's disease, in spite of the fact that there was marked impairment of liver function in one (J. St.) and moderate impairment in the other (C. L.).

TABLE IV

Radioactivity in urine and stools following the intravenous administration of radiocopper

Group	Patient	Per cent of administered activity		
		Urine	Stool	Total
Normal subjects	O. H.	0.2	9.3	9.5
	D. Ca.	0.3	16.1	16.4
	A. P.	0.2	14.2	14.4
	R. W.	0.3	9.9	10.2
	Mean	0.2	12.4	12.6
Hepatolenticular degeneration	D. C.	3.2	2.3	5.5
	B. S.	5.2	1.8	7.0
	J. Si.	7.8	3.4	11.2
	Mean	5.4	2.5	7.9
Alcoholic hepatic cirrhosis	J. St.	1.0	7.0	8.0
	C. L.	0.7	9.9	10.6
	Mean	0.8	8.5	9.3

Plasma activity following oral administration of radiocopper

The activity in whole plasma was studied in four normal subjects and in all four of the patients with Wilson's disease given radiocopper by mouth (Figure 2). The plasma of one normal subject (S. F., Figures 3 and 4) and of one patient with Wilson's disease (J. Si., Figure 4) was fractionated by means of both anti-ceruloplasmin serum and ammonium sulfate. The plasma of one patient (B. S., Figure 3) was fractionated only with anti-serum; whereas, the plasma samples of one normal subject (D. L.) and one patient with hepatolenticular degeneration (D. C.) were fractionated only with ammonium sulfate (Figure 4).

Following the oral administration of radioactive copper, there was a prompt increase in activity in the plasma of both the normal subjects and the patients with Wilson's disease. The peak value in all subjects occurred between one and three hours after ingestion of the isotope, and this tended to be higher in the patients with Wilson's disease than in the normal subjects. However, in two of the normal subjects (S. F. and R. H.), the values were equal to the two lowest in the patient group (D. C. and J. Si.). There was poor correlation (correlation coefficient + 0.48, $P = 0.25$) between the peak value for plasma activity and the amount of radioactivity absorbed and retained (100 − stool and urine activity) (Tables II and III).

In the normal subjects, the activity in the plasma decreased rapidly during the 4 to 6 hours following maximal activity, and thereafter increased slowly. On the other hand, in the patients with Wilson's disease, the activity declined more slowly than in the control subjects and no secondary increase was observed.

The activity of the plasma during the initial peak in both the normal subjects and the patients was almost entirely in the supernatant solution remaining after precipitation with anti-ceruloplasmin serum (Figure 3). In the normal subjects, incorporation of radiocopper into the precipitate (ceruloplasmin) could be detected within two hours after the administration of radiocopper and the radioactivity of this fraction increased with

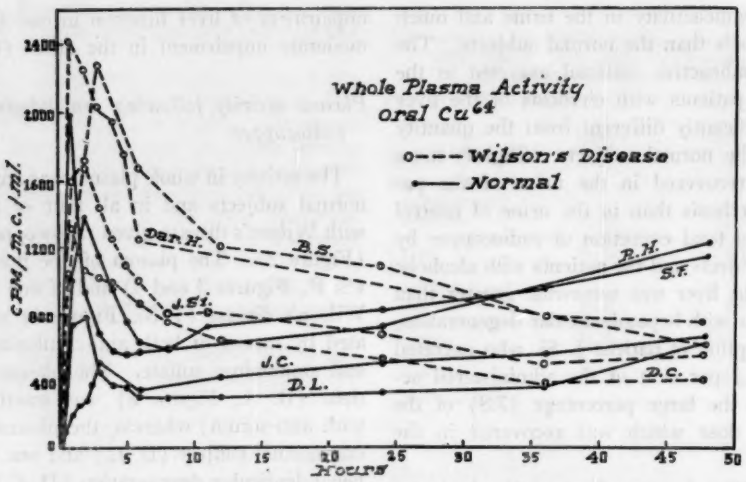

FIG. 2. WHOLE PLASMA ACTIVITY FOLLOWING ORAL ADMINISTRATION OF RADIOCOPPER
The values on the ordinate refer to CPM/mc. of ingested radiocopper in one ml. of whole plasma.

time. As a result, the secondary rise in whole plasma activity (Figure 2) was due to the appearance of radioactivity in the anti-serum precipitate (ceruloplasmin) (Figures 3 and 4). In contrast, no measurable quantities of radiocopper were incorporated into the precipitate (ceruloplasmin) in the two patients with Wilson's disease (B. S. and J. Si.), and no secondary rise in whole plasma activity occurred.

When plasma was fractionated with ammonium sulfate, activity was present in the precipitate from the plasma of the patients with Wilson's disease. By comparison of the activity in the ammonium sulfate precipitate with the activity in

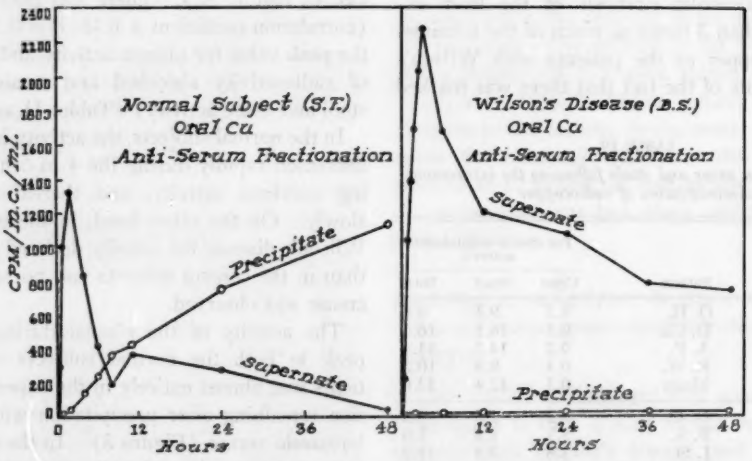

FIG. 3. RADIOACTIVITY IN FRACTIONS OF PLASMA PRODUCED BY ADDITION OF RABBIT ANTI-HUMAN CERULOPLASMIN SERUM
The values on the ordinate refer to CPM/mc. of radiocopper ingested appearing in fractions derived from one ml. of plasma.

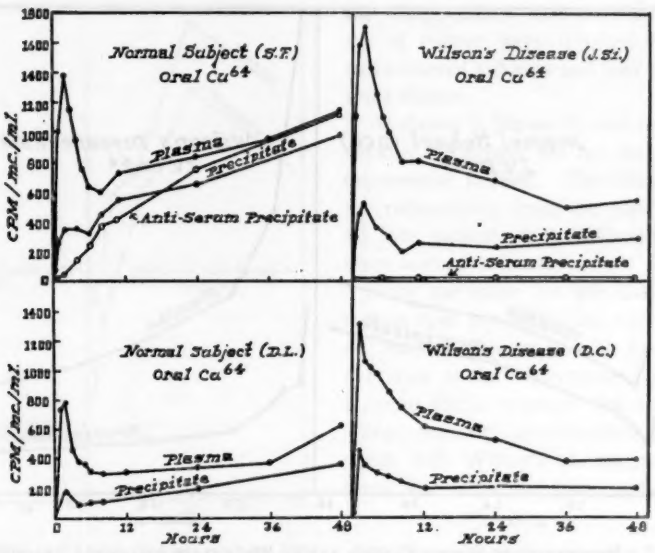

FIG. 4. RADIOACTIVITY IN WHOLE PLASMA AND IN FRACTIONS PRODUCED BY ADDING AMMONIUM SULFATE (50 PER CENT SATURATION)

For purposes of comparison, the activity in the precipitate after the addition of rabbit anti-human ceruloplasmin serum in one normal subject (S. F.) and in one patient with Wilson's disease (J. Si.) is also shown. The difference between the curves of activity in whole plasma and in the ammonium sulfate precipitate represents the activity in the supernate after 50 per cent saturation with ammonium sulfate. The values on the ordinate refer to CPM/mc. of ingested radiocopper in one ml. of whole plasma or in the precipitate obtained from one ml. of plasma.

the precipitate after the addition of anti-serum it can be seen that the activity in the former was due to non-ceruloplasmin copper which was carried down with the precipitate (Figure 4).

Plasma activity following intravenous administration of radiocopper

The activity of whole plasma was measured in three normal subjects, four patients with hepatolenticular degeneration, and two patients with alcoholic cirrhosis of the liver. The plasma of two patients in each group was fractionated with ammonium sulfate. The results are presented in Figures 5, 6, and 7.

The activity of whole plasma in the normal subjects (Figures 5 and 6) decreased rapidly during the first three hours after injection. Thereafter, the activity increased progressively with time. During the first three hours the activity was almost entirely in the supernatant solution after fractionation with ammonium sulfate. Thereafter, the activity in the precipitate after ammonium sulfate fractionation increased progressively and most of the activity of the whole plasma was accounted for in this fraction.

In the patients with Wilson's disease (Figures 5 and 6), the activity of the whole plasma decreased more slowly than in the normal subjects and a secondary rise in activity was not observed. The activity in the precipitate from ammonium sulfate fractionation was high during the first hours following the injection (due to non-ceruloplasmin copper carried down with the precipitate [Figures 1 and 4]) and decreased rapidly at first and then more slowly. A progressive increase in the activity in the precipitate, such as was observed in the normal subjects, did not occur.

In one of the patients (J. St., Figure 7) with alcoholic cirrhosis of the liver and a normal plasma

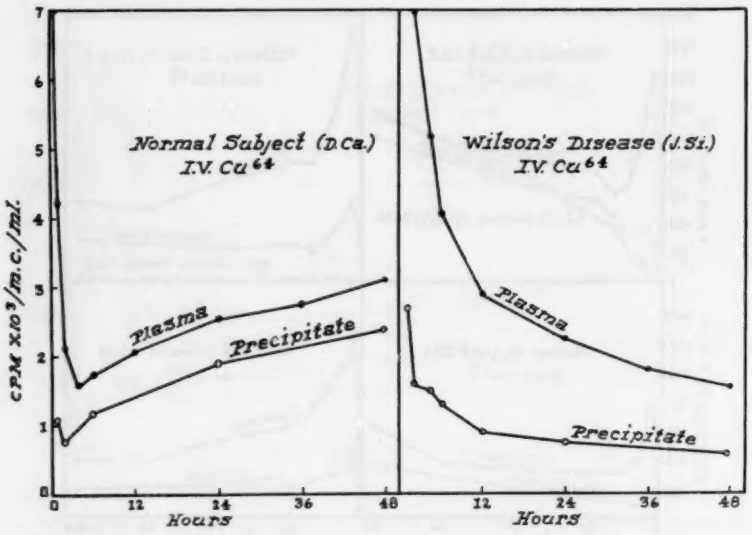

FIG. 5. RADIOCOPPER IN WHOLE PLASMA AND IN PRECIPITATE FOLLOWING FRACTIONATION WITH AMMONIUM SULFATE (50 PER CENT SATURATION)

The difference between the curves of activity in whole plasma and in the ammonium sulfate precipitate represents the activity in the supernate. The values on the ordinate refer to CPM/mc. of injected radiocopper in one ml. of whole plasma or in the precipitate obtained from one ml. of plasma.

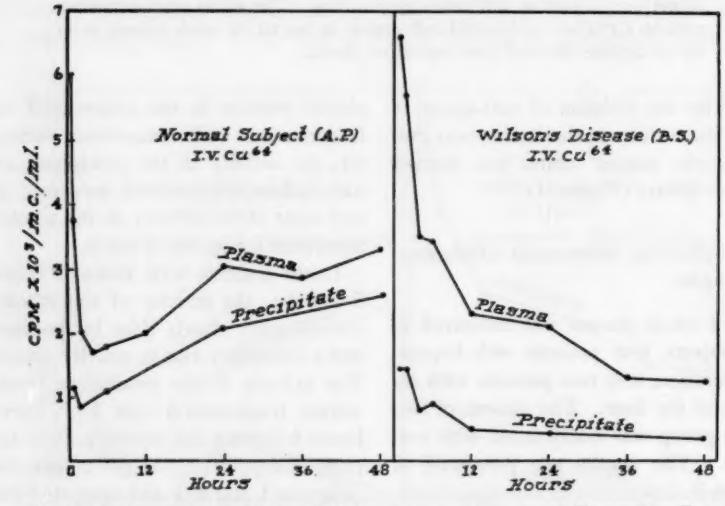

FIG. 6. RADIOCOPPER IN WHOLE PLASMA AND IN PRECIPITATE FOLLOWING FRACTIONATION WITH AMMONIUM SULFATE (50 PER CENT SATURATION)

The difference between the curves of activity in whole plasma and in the ammonium sulfate precipitate represents the activity in the supernate. The values on the ordinate refer to CPM/mc. of injected radiocopper in one ml. of whole plasma or in the precipitate obtained from one ml. of plasma.

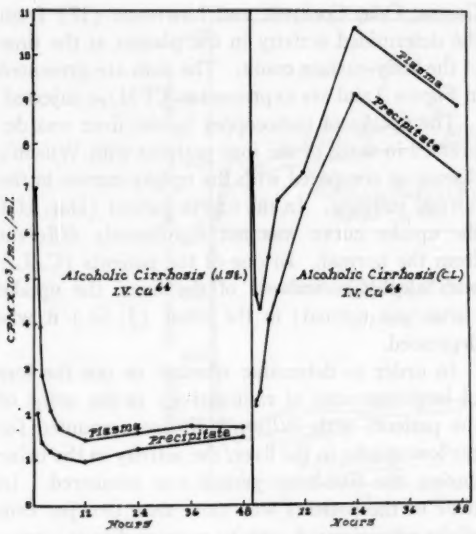

FIG. 7. RADIOCOPPER IN WHOLE PLASMA AND IN PRECIPITATE FOLLOWING FRACTIONATION WITH AMMONIUM SULFATE (50 PER CENT SATURATION)

The difference between the curves of activity in whole plasma and in ammonium sulfate precipitate represents the activity in the supernate. The values on the ordinate refer to CPM/mc. of injected radiocopper in one ml. of whole plasma or in the precipitate obtained from one ml. of plasma.

copper level (110 μg. per cent), the pattern did not differ from that observed in the normal subjects. The second patient (C. L., Figure 7) had hypercupremia (170 μg. per cent) and there was a striking increase in the activity in the whole plasma beginning two hours after the injection. This secondary increase was due entirely to increased activity in the precipitate.

The disappearance of radiocopper from plasma

The rate of disappearance of radioactivity from whole plasma after the oral (Figure 2) or intravenous (Figures 5 and 6) administration of one mg. of radiocopper was slower in the patients with hepatolenticular degeneration than in the normal subjects. In order to determine if the radiocopper disappeared according to a first order equation, the data were plotted on semi-logarithmic paper. A straight line was not obtained in either group. In these studies a relatively large dose of copper (1 mg.) was employed. In order to study the disappearance rate after a "tracer dose," 50 μg. of copper were injected intravenously into three normal subjects and four patients with Wilson's disease.

As shown in Figure 8, even under these circumstances, the copper did not disappear in a simple exponential fashion. The disappearance rate of the radioactivity from the plasma was slower in all four patients with Wilson's disease than in three normal subjects.

Since the curve for the disappearance rate of copper does not follow the equation of a first order reaction, it is not possible to calculate the turnover rate of the "direct-reacting" plasma copper. It seems likely, however, that since the amount of "direct-reacting" or albumin-bound copper in patients with Wilson's disease was at least three times that in normal subjects (3), the amount of

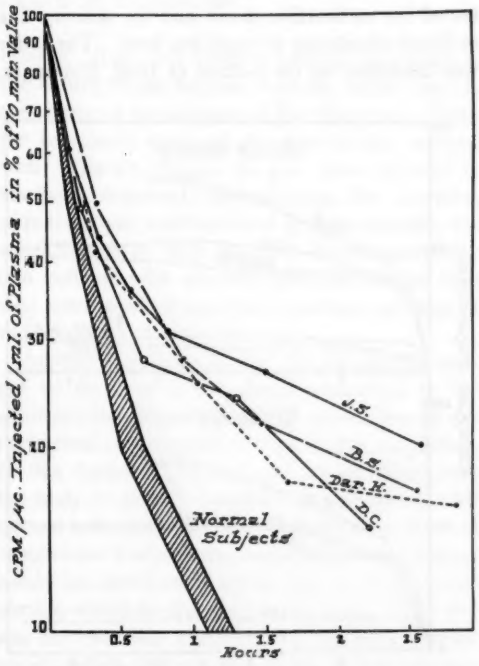

FIG. 8. DISAPPEARANCE RATE OF INTRAVENOUSLY ADMINISTERED RADIOCOPPER (50 μg.) FROM PLASMA OF NORMAL SUBJECTS AND PATIENTS WITH WILSON'S DISEASE

The value obtained 10 minutes after the intravenous injection was taken as 100 per cent. Subsequent values are plotted as per cent of the activity in the 10-minute specimen.

"direct-reacting" plasma copper turned over per unit of time may have been equal to or greater than the amount in the normal subjects, even though the disappearance rate of radiocopper was slower in the former than in the latter.

The uptake of radiocopper by liver

The uptake of radiocopper by the liver was estimated by the use of a mobile body-surface scintillation counter placed over the liver. This determination was performed following the intravenous administration of 50 μg. of radioactive copper to two normal subjects, four patients with Wilson's disease, and two patients with alcoholic cirrhosis of the liver. Prior to the injection of the isotope, the point of maximal liver dullness was determined and marked with ink. Care was taken to place the counter in the same position at the time of each counting. The determined counts were corrected for radioactive decay and for activity due to blood circulating through the liver. The latter was calculated by the method of Huff, Elmlinger, Garcia, Oda, Cockrell, and Lawrence (17) from the determined activity in the plasma at the time of the body-surface count. The data are presented in Figure 9 and are expressed as CPM/μc injected.

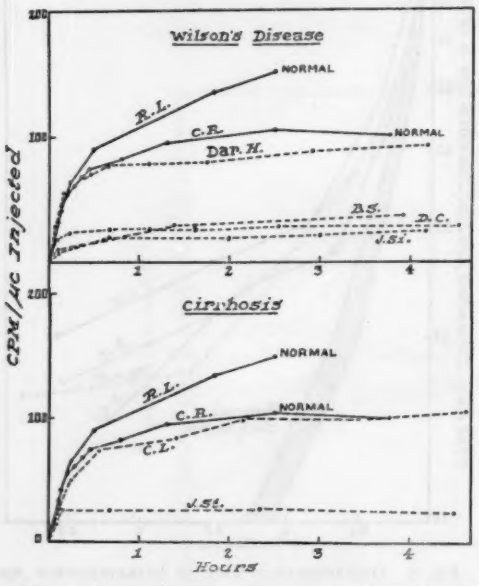

FIG. 9. UPTAKE OF RADIOCOPPER BY LIVER OF NORMAL SUBJECTS (R. L. AND C. R.), PATIENTS WITH WILSON'S DISEASE (DAR. H., B. S., D. C., AND J. ST.), AND PATIENTS WITH CIRRHOSIS OF LIVER (C. L. AND J. ST.) AS MEASURED WITH MOBILE BODY-SURFACE SCINTILLATION COUNTER

The uptake of radiocopper by the liver was depressed in three of the four patients with Wilson's disease as compared with the uptake curves in the normal subjects. In the fourth patient (Dar. H.) the uptake curve was not significantly different from the normal. In one of the patients (C. L.) with alcoholic cirrhosis of the liver, the uptake curve was normal; in the other (J. St.) it was depressed.

In order to determine whether or not the loss of large amounts of radioactivity in the urine of the patients with Wilson's disease accounted for the low uptake in the liver, the activity in the urine during the five-hour period was measured. In none of the patients was more than two per cent of the administered activity recovered in the urine during the period of study.

In an effort to localize the major portion of the injected copper in the patients with hepatolenticular degeneration, the counter was placed over the skull, sacrum, lungs, heart, spleen, and kidney. No localized accumulation of radioactivity could be detected in any of these sites.

The uptake of radiocopper by erythrocytes

The uptake of radiocopper by erythrocytes was studied in four normal subjects and in four patients with Wilson's disease. Representative results in two normal subjects and in two patients are presented in Figure 10.

The activity in one ml. of whole blood and in one ml. of plasma was measured. The differences between these values were considered to represent the activity in one ml. of packed erythrocytes after appropriate correction for relative plasma and red cell volume as determined by the volume of packed red cells. The red cell values have not been corrected for "trapped plasma" and are expressed in CPM per ml. of plasma or red cell water, on the assumption that the plasma contained 90 per cent water and the erythrocytes 64 per cent water.

Following the oral administration of radiocopper there was a rapid uptake of activity by the red cells in both the normal subjects and the patients with Wilson's disease. Thereafter there was a

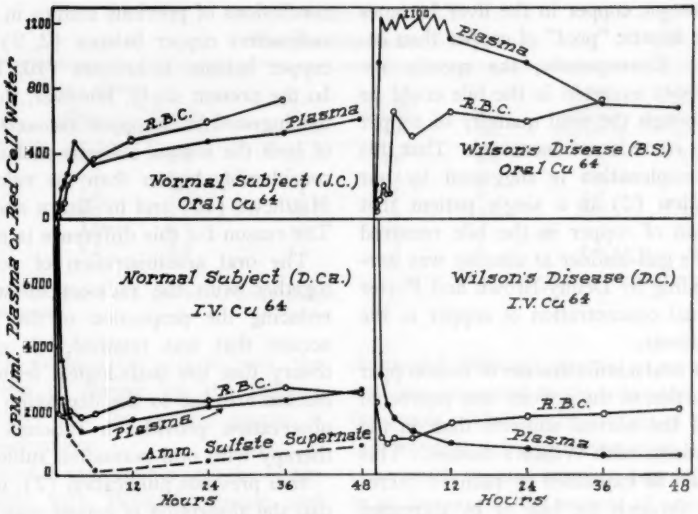

FIG. 10. THE UPTAKE OF RADIOCOPPER BY ERYTHROCYTES

slow progressive increase in the activity in the cells. Similarly, following the intravenous administration of the isotope, considerable activity was found in the erythrocytes in 10 minutes. During the next two hours, as the plasma activity declined rapidly, there was a decline in the activity in the cells. Thereafter, the activity in the red cells increased slowly despite the fact that the activity in the supernatant solution after ammonium sulfate fractionation remained low in the normal subjects.

DISCUSSION

The proportion of the intravenously administered radiocopper which was recovered in the stools of the patients with hepatolenticular degeneration was distinctly less than the amount recovered in the stools of the normal subjects. A similar observation has been made by Bearn and Kunkel (13). Two explanations for these results seem reasonable to us. Since it has been demonstrated in dogs (18) that the main excretory route for copper is through the biliary system,[5] it follows that the ability of the liver to excrete copper into the bile may be impaired in Wilson's disease. If so, this defect appears to be related to the nature of the hepatic disorder rather than to the degree of impairment of liver function. Thus, the functional status of the liver of the patients with Wilson's disease ranged from normal to markedly impaired; nevertheless, the excretion of radiocopper was depressed to approximately the same degree in each patient. Furthermore, the two patients with alcoholic cirrhosis of the liver who were studied excreted a normal quantity of radiocopper.

Another possibility is that the excretion of copper by the liver is not actually impaired in hepatolenticular degeneration. There are several experimental observations to support this suggestion. In this disease the quantity of copper throughout the body is greatly increased as compared with normal individuals (2, 8). Consequently, it could be expected that a given amount of isotopic copper would be distributed widely and a smaller proportion would be deposited in the liver. That this was the case was suggested by the body surface counts which showed that three of our patients with Wilson's disease did not concentrate the isotope in the liver to the same degree as did normal subjects.

In addition to the likelihood that a smaller proportion of radiocopper is carried to the liver in the cases of Wilson's disease than in the normal

[5] We have recently given radiocopper intravenously to a patient with complete obstruction of the bile duct and a cutaneous bile fistula. In 72 hours, 7.8 per cent of the radioactivity was recovered in the bile; 0.8 was recovered in the urine; and 2.1 per cent in the stools.

subjects, the isotopic copper in the liver becomes part of a larger hepatic "pool" of copper than occurs normally. Consequently, the specific activity of the copper excreted in the bile could be reduced even though the total quantity of copper excreted by this route is not decreased. That this is a plausible explanation is suggested by our earlier observation (2) in a single patient that the concentration of copper in the bile removed directly from the gall-bladder at autopsy was normal and the finding by Denny-Brown and Porter (19) of a normal concentration of copper in the bile of three patients.

Following the oral administration of radiocopper a greater proportion of the activity was recovered in the stools of the normal subjects than in the stools of the patients with Wilson's disease. This observation could be explained by reduced excretion of isotope through the bile or by increased absorption in the patients with Wilson's disease. Although the facts are unknown, if it were assumed that radiocopper which is absorbed from the gastro-intestinal tract is handled in the same manner as intravenously injected copper, this question lends itself to mathematical solution, thus:

$$x = \frac{100\,A}{100 - E}$$

x is the per cent of ingested radiocopper absorbed,

A is equal to 100 minus the per cent of radiocopper recovered in the stool,

E is the sum of the per cent of copper excreted through the kidneys, liver, and intestinal wall following the intravenous administration of radiocopper.

According to this calculation the normal subjects absorbed an average of 32 per cent (6 to 43) of the ingested radiocopper and the patients absorbed an average of 49 per cent. Three patients with Wilson's disease absorbed 55, 68, and 52 per cent. A fourth patient (J. Si.) absorbed only 20 per cent. Thus, it appears that three of the four patients with Wilson's disease absorbed more radiocopper from the gastrointestinal tract than did the normal subjects.

The observation that the intestinal absorption of copper appeared to be increased in patients with Wilson's disease is in agreement with the conclusions of previous studies in which the nonradioactive copper balance (2, 9) and the radiocopper balance techniques (10, 13) were used. In the present study, however, the proportion of the ingested radiocopper recovered in the stools of both the normal subjects and the patients was considerably higher than the values obtained by Matthews (10) and by Bearn and Kunkel (13). The reason for this difference is not apparent.

The oral administration of potassium sulfide together with the radiocopper was effective in reducing the proportion of the ingested cupric acetate that was retained. If one accepts the theory that the pathological lesions of Wilson's disease are due to the deposition of copper, this observation provides a rationale for long-term therapy with oral potassium sulfide.

In a previous publication (2), it was suggested that the absorption of copper may be increased in this disease because the ceruloplasmin level is decreased. This theory is not supported by our present data since D. C., with ceruloplasmin level of 19 mg. per 100 ml., absorbed about 68 per cent of the radiocopper; B. S., with a level of 2 mg. per 100 ml., absorbed 55 per cent; and J. Si., with a level of 4 mg. per 100 ml., absorbed 20 per cent.

The uptake of radiocopper into ceruloplasmin was greatly impaired in the patients with Wilson's disease. A similar observation has been made by Bearn and Kunkel (13), Earl, Moulton, and Selverstone (15), and Jensen (20). This could be due either to an impaired rate of ceruloplasmin synthesis or to dilution of the isotope in a large pool of copper before it was incorporated into ceruloplasmin. Since the uptake of radiocopper into ceruloplasmin was no greater in patient D. C. (Figure 4) with a ceruloplasmin concentration of 19 mg. per 100 ml. than in patient J. Si. (Figure 4) with 4 mg. per 100 ml., it seems likely that deficient ceruloplasmin in the plasma is not the only explanation for the low uptake.

The finding that the albumin-bound (direct-reacting) copper is markedly increased in Wilson's disease confirms our earlier observations (2) and the observations of others (7, 13, 15). The increase in this fraction is probably a reflection of the extremely high tissue levels of copper, and the increased absorption of copper from the gastro-intestinal tract. The high concentration of this loosely bound copper, as suggested by Bearn

TABLE V

Liver function studies

Patient	Plasma protein			B.S.P.*	Thymol turbidity	Ceph. floc.	Total plasma bilirubin	Alkaline phosphatase	Prothrombin time
	Total gm./100 ml.	Albumin gm./100 ml.	Globulin gm./100 ml.	% retention	units		mg./100 ml.	units†	% of normal
D. C.	6.6	3.8	2.8	35	13	4+	3.2	10	59
B. S.	6.3	4.7	1.6	5	3	0	0.8	5	79
J. Si.	6.0	3.8	2.2	5	1	0	0.9	1	100
Dar. H.	6.8	4.7	2.1	10	6	3+	0.7	12	100
J. St.	7.7	3.0	4.7	40	13	4+	3.6	8	40
C. L.	8.6	5.1	3.5	17	5	0	1.4	6	100

* Forty-five minutes after 5 mg. per kg. of body weight.
† King and Armstrong.

and Kunkel (13), explains, in part at least, the increased excretion of copper via the kidneys since it seems likely that the urinary copper is derived from this fraction.

The uptake curves of radiocopper into the erythrocytes of patients with Wilson's disease and normal subjects were similar after the first 12 hours. The incorporation of copper into normal erythrocytes is now being investigated in detail and the results will be reported later.

SUMMARY

1. One mg. of copper containing 1 mc. of radioactivity was administered orally to four normal subjects and four patients with Wilson's disease. The same amount of radiocopper was injected intravenously into four normal subjects, three patients with Wilson's disease, and two patients with alcoholic cirrhosis of the liver. The proportion of the administered radiocopper excreted in the urine and feces was determined. In addition, the uptake of the isotope into the albumin and globulin (ceruloplasmin) fractions of plasma was studied. After the administration of a dose of 50 μg. of radiocopper, the curve of disappearance of Cu^{64} from the plasma was determined in three normal subjects, four patients with Wilson's disease, and two patients with alcoholic cirrhosis of the liver. The uptake of radiocopper into the liver was determined with a body-surface scintillation counter in two normal subjects, four patients with hepatolenticular degeneration, and two patients with alcoholic cirrhosis of the liver.

2. Following the oral administration of radiocopper to normal subjects, an average of 0.1 per cent of the administered dose was recovered in the urine and 72.4 per cent in the stools; in the patients with Wilson's disease, an average of 2.5 per cent was recovered in the urine and 52 per cent in the stools. The administration of potassium sulfide together with radiocopper to two of the patients with hepatolenticular degeneration resulted in an increase in the amount of activity recovered in the stools.

3. Following the intravenous administration of radiocopper to normal subjects, an average of 0.2 per cent of the activity was recovered in the urine and 12.4 per cent in the stools; in the patients with Wilson's disease, an average of 5.4 per cent was recovered in the urine and 2.5 per cent in the stools. In the patients with alcoholic cirrhosis of the liver, an average of 0.8 per cent was recovered in the urine and 8.5 per cent in the stools.

4. The uptake of radiocopper into ceruloplasmin was impaired in the patients with hepatolenticular degeneration as compared with that in normal subjects. The uptake was not impaired in the patients with alcoholic cirrhosis of the liver.

5. The uptake of radiocopper by the liver, as measured by a body-surface scintillation counter, was depressed in three of the four patients with hepatolenticular degeneration and in one of two patients with alcoholic cirrhosis of the liver, as compared with the uptake in two normal subjects.

6. The uptake of radiocopper by the erythrocytes of patients with hepatolenticular degeneration was not different from that of the red corpuscles of normal individuals.

7. These findings are interpreted as indicating that the excessive accumulation of copper in the tissues of patients is due primarily to increased absorption of copper from the gastro-intestinal tract. Although a smaller proportion of the isotope administered intravenously was recovered in

the stools of the patients with Wilson's disease than was recovered in the stools of normal subjects, evidence has been presented in support of the concept that the excretion of copper in the bile may not be impaired.

8. The absorption of cupric acetate can be effectively inhibited by the administration of potassium sulfide.

ACKNOWLEDGMENTS

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THE PHYSIOLOGICAL DISPOSITION AND METABOLIC FATE OF HYDROCORTISONE IN MAN

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The present report is concerned primarily with the physiological disposition and fate of hydrocortisone in man. Large doses of this steroid have been administered intravenously, and the rate of its disappearance from plasma has been determined in normal subjects and in patients with liver disease and various endocrinopathies. Trace quantities of hydrocortisone-4-C^{14} have been employed in certain studies, either alone or as a label for the larger doses. From these studies information has been obtained regarding rates of metabolic transformation, rates and routes of excretion of hydrocortisone and its metabolites, and to some extent the identity and amounts of these metabolites. Data on the rate and extent of absorption of hydrocortisone after oral administration have also been obtained. Since, in most respects, the results obtained upon administering large or trace quantities of hydrocortisone did not differ greatly, the findings are to a considerable extent also applicable to problems of endogenous hydrocortisone metabolism.

MATERIALS AND METHODS

Administration of steroid

Fifty to 500 mg. of hydrocortisone were dissolved in ethanol and added to 500 ml. of sterile 5 per cent dextrose in water, producing a final alcohol concentration of 2 to 3 per cent. This solution was given intravenously over a period of 20 to 30 minutes. Following the infusion, blood samples were drawn in heparinized syringes every 20 to 30 minutes for 2 to 3 hours. Urine samples were collected for 24 hours or longer in single or fractional specimens, and preserved by freezing.

Procedure for determination of plasma hydrocortisone

The following procedure is a modification of the recently published method of Silber and Porter (1):

Principle—Hydrocortisone is extracted from plasma into dichloromethane. The dichloromethane extract is washed with aqueous alkali to remove a considerable amount of "blank" material. The dichloromethane is then shaken with a sulfuric acid-ethanol reagent, containing phenylhydrazine. The resulting colored product is measured in the acid phase spectrophotometrically at 410 mμ. A correction for material in plasma reacting with sulfuric acid is made by treating an equal aliquot of dichloromethane extract of plasma with sulfuric acid-ethanol which contains no phenylhydrazine.

Reagents.

(a) *Dichloromethane*—This solvent is purified by passing through a bed of silical gel (average 100 mesh) in a 7 by 125 cm. column. Ten to twenty liters can be purified in 2 to 3 hours. The effectiveness of purification is determined by shaking an aliquot of solvent with the phenylhydrazine-sulfuric acid-ethanol reagent (f). No color should develop on standing overnight at room temperature. The solvent has remained free of impurities for many months at room temperature.

(b) *0.1 N Sodium Hydroxide.*

(c) *70 Per Cent Sulfuric Acid*—700 ml. acid C.P. grade sulfuric acid added to 300 ml. H_2O.

(d) *Ethanol*—This is purified by adding 7 gm. silver nitrate and 15 gm. potassium hydroxide, separately (each dissolved in 100 ml. ethanol) to 4 liters of absolute ethanol. These are mixed, allowed to stand overnight, and then distilled, using a Vigreaux column. The first 700 ml. and the last 100 ml. portions are discarded. The effectiveness of purification is determined by reacting this ethanol with the phenylhydrazine-sulfuric acid (f). No color should develop on standing overnight at room temperature.

(e) *Blank Reagent*—Two parts (c) with one part (d).

(f) *Phenylhydrazine-Sulfuric Acid-Ethanol Reagent*—50 mg. of phenylhydrazine hydrochloride (recrystallized from ethanol and water) are dissolved in 50 ml. of the blank reagent (e).

(g) *Hydrocortisone Standard*—100 mg. of hydrocortisone are dissolved in 100 ml. absolute ethanol. A working standard is made by diluting one ml. to a volume of 100 ml. with water (10 micrograms per ml. water).

Procedure. (All glassware used *must be scrupulously cleaned* with concentrated sulfuric acid.)

1. *Extraction*—Carefully add 5 ml. plasma to 25 ml. dichloromethane in a 200-ml. Erlenmeyer flask and place on rotating table (Arthur H. Thomas No. 3623). Extract for 10 to 15 minutes with gentle rotation (this procedure extracts more than 98 per cent of the hydrocortisone, free and protein bound, in plasma). *Gently* transfer entire contents to a 25-ml. graduated cylinder.

2. *Washing*—After aspirating as much plasma as possible, add 2 ml. 0.1 N sodium hydroxide to dichloromethane extract, shake for 15 to 20 seconds, and then remove the alkali layer by aspiration.

3. *Aliquots*—Transfer two 10-ml. aliquots (for unknown and blank) and place in separate 15-ml. ground glass stoppered conical test tubes.

4. *Color Development*—For unknowns, add 0.2 ml. phenylhydrazine-sulfuric acid-ethanol reagent (f) to dichloromethane extract aliquot; for blanks, add 0.2 ml. blank reagent (e). Stopper the tubes, shake vigorously for 15 to 20 seconds, and allow to stand for 30 minutes or more. The supernatant dichloromethane phase is removed by aspiration and the sulfuric acid-ethanol solution allowed to stand at room temperature for 10 or more hours for maximum color development.

5. *Spectrophotometry*—Transfer the contents of the tubes to microcuvettes (Pyrocell, 1.5 by 10 by 15 mm.) and measure the absorbency of the colored product against a water blank at 410 mμ in Beckman DU spectrophotometer.

Note: 5 ml. water run through the entire procedure serves as reagent blank, and 5 ml. water containing 5 micrograms of hydrocortisone serves as standard.

Specificity of procedure for determination of hydrocortisone in plasma after administration of hydrocortisone—Specificity of the method was shown by counter-current distribution. Plasma drawn 90 minutes after the end of a 30-minute intravenous infusion of 200 mg. of hydrocortisone was extracted with dichloromethane, the extract evaporated to dryness, and the apparent hydrocortisone subjected to an eight-transfer counter-current distribution. The solvents used were water (8 volumes) and chloroform (1 volume). This system gave a partition coefficient of about 0.1 for authentic hydrocortisone. A trace quantity of hydrocortisone-4-C^{14} was added before the counter-current distribution. After distribution, aliquots of both phases were analyzed for carbon^{14} and by the phenylhydrazine procedure. Figure 1 demonstrates the distribution of the phenylhydrazine reacting material and the carbon^{14} hydrocortisone. These data demonstrated that more than 90 per cent of the material assayed with phenylhydrazine was distributed as hydrocortisone.

The specificity of the analytical procedure employed was further demonstrated by subjecting dichloromethane extracts of plasma obtained 60 and 120 minutes following the end of a 30-minute infusion of 200 mg. of hydrocortisone to paper chromatography, using a modification of the Bush (2) type system. A small quantity of hydrocortisone-4-C^{14} was added to the dichloromethane extract and the specific activity of the phenylhydrazine reacting material was determined. The extract was then chromatographed at room temperature for 6 hours in a benzene-methanol-water (4:2:1) system. The hydrocortisone of the extract, running at the same rate as pure hydrocortisone, was located by ultraviolet scanning. This area was cut out and eluted with 3 ml. of 95 per cent ethanol, and the eluate evaporated to dryness under nitrogen. The residue was redissolved and the specific ac-

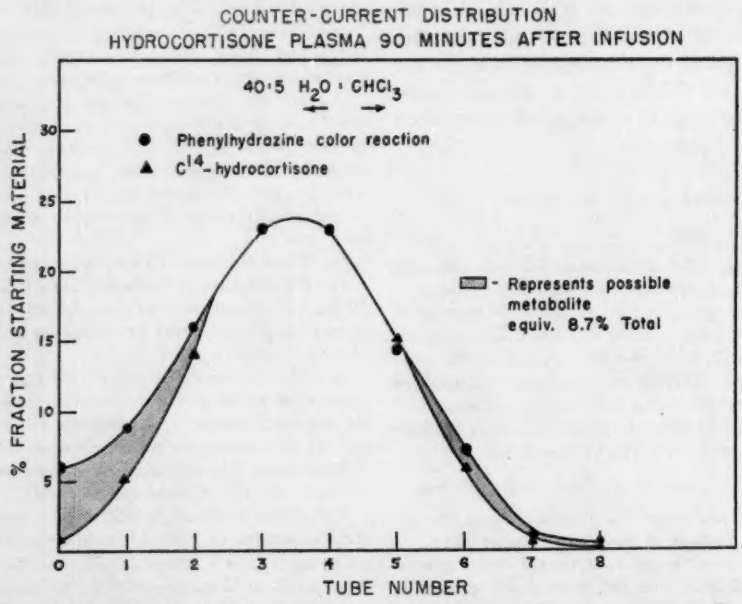


FIG. 1. COUNTER-CURRENT DISTRIBUTION ANALYSIS OF PHENYLHYDRAZINE REACTING MATERIAL IN PLASMA DICHLOROMETHANE EXTRACT 90 MINUTES FOLLOWING TERMINATION OF INFUSION OF 200 MG. HYDROCORTISONE TO NORMAL SUBJECT

tivity of the phenylhydrazine reacting material was again determined. Table I shows the specific activity of the material before and after chromatography. Portions of the steroid eluted from the paper were also assayed fluorometrically by a modification of the highly specific method described by Sweat (3). Other eluted portions were treated so as to convert the hydrocortisone to its acetate which was then chromatographed, eluted, and assayed. These procedures confirmed the data obtained by counter-current distribution studies.

Procedure for determination of hydrocortisone metabolites in urine—The method and reagents were the same as those described for plasma, except that to the urine (10 ml.) 2 ml. 0.5 M phosphate buffer (pH 6.2) and 1000 units Sigma bacterial glucuronidase in one ml. of water were added. Following incubation for 24 hours at 37° C., the mixture was extracted with 8 to 9 volumes of dichloromethane. After washing the dichloromethane extract with 1/20 volume 0.1 N NaOH, aliquots (40 ml.) were extracted with 3.0 ml. of the phenylhydrazine-sulfuric acid-ethanol, and the sulfuric acid-ethanol reagent as described for plasma. The resulting colored product was measured at 410 mμ.

Phenylhydrazine reacting materials extracted from urine either directly or after β-glucuronidase hydrolysis represent many different metabolites of hydrocortisone.

Studies with hydrocortisone-4-C^{14}

Hydrocortisone-4-C^{14}, specific activity 1.47 millicuries per millimole, was made available through the Endocrine Study Section of the National Institutes of Health. This compound was shown to be approximately 95 per cent pure by counter-current distribution, and isotope dilution analyses and paper chromatography. It was administered intravenously in trace quantities in solution in a small volume of 5 per cent ethanol in water over a period of 3 to 5 minutes, or mixed with non-isotopic hydrocortisone given in 500 ml. 3 per cent ethanol in 5 per cent dextrose in sterile distilled water. Similar solutions were administered orally in certain studies.

After intravenous or oral administration, samples of blood were taken at 30 to 60-minute intervals for 4 to 8 hours, and urine was collected every 2 hours for 12 hours, the following 12 hours, and for 24-hour periods until no additional radioactivity was demonstrable. Fecal samples were collected for 4 days, homogenized with water in a Waring blendor, and an aliquot lyophilized.

Plasma samples were extracted directly with 5 volumes of dichloromethane. The dichloromethane extract was washed once with 1/20 volume 0.1 N sodium hydroxide, twice with 1/15 volume water, and then evaporated to dryness at 40° C. under nitrogen. The residue was then dissolved in methanol and an aliquot added to the planchet.

Biliary excretion of radiometabolites of hydrocortisone—Bile collections were made on a 53-year old male patient who gave a history of intermittent colicky pain of several years' duration with signs of recurrent obstructive jaundice for 8 months, and persistent signs of jaundice and RUQ pain for 3 weeks prior to hospitalization.

TABLE I

Comparison specific activities phenylhydrazine reacting material in plasma extracts following infusion of hydrocortisone

	Specific activities (cpm/μg.)	
	Before chromatography	After chromatography
Expt. No. 1 (plasma 60 min. after infusion)		
1st chromatography	2,370	2,680
2nd chromatography		2,710
3rd chromatography*		2,500
Expt. No. 2 (plasma 120 min. after infusion)		
1st chromatography	635	650†

* Converted to hydrocortisone acetate and rechromatographed.

† Six hundred and fifty cpm per microgram with phenylhydrazine and fluorometric assay.

Liver function tests were compatible with a diagnosis of obstructive jaundice. Surgical exploration[1] revealed the presence of cholecystitis and chololithiasis, and in addition revealed an acute pancreatitis completely obstructing the common bile duct which was dilated to 3 to 4 times its normal size. No stones were found on exploration of the common bile duct. The sphincter of Oddi was cut. A T-tube was inserted in the common duct, and at its distal end was attached a small "Foley-type" catheter. These two tubes were led to the outside. Bile was permitted free access to the T-tube and to the duodenum through the common duct; however, the distal end of the common duct could be obstructed at will by inflating the Foley catheter bag with 1 to 2 ml. water, thereby draining all the bile through the T-tube. On the morning of the study, the bile duct was obstructed and the patient was given 1.5 μc. of hydrocortisone-4-C^{14} intravenously. Bile samples were collected every two hours for 12 hours, and for the following 12 hours. Feces and urine were also collected.

Procedure for carbon^{14} analyses of samples—All analyses for carbon^{14} were carried out with a Robinson (4) gas-flow counter, using a gas mixture of nine parts argon and one part methane, and connected to a Nuclear Model 172 scaler. This apparatus gave a background of 3 to 4 cpm and an efficiency for carbon^{14} of slightly more than 53 per cent when counted at infinite thinness. Planchets of stainless steel with a surface area of 1.6 sq. cm. and a depth of 0.6 cm. were used. They were cleaned by boiling with alcoholic potassium hydroxide.

From 0.05 to 0.30 ml. of fresh urine was plated directly onto tared planchets. The urine was dried on a rotating platform with the aid of air and heat from an infrared

[1] We are indebted to Dr. Henry Doubilet of New York University College of Medicine, and to Dr. Robert Smith, National Cancer Institute, for the operative procedures involved in carrying out this study.

lamp, and the planchets were then placed in a desiccator until weighed and counted. A correction for self-absorption was made from a self-absorption curve of urine, and all samples were corrected to infinite thinness. Self-absorption curves run on different urines were not found to show a significant degree of variation. It was not necessary to correct any samples for self-absorption that weighed less than 0.1 mg. (infinite thinness). Results obtained using a double isotope dilution technique (5) were found to check with results obtained with the self-absorption correction curve data. All counting results were corrected for instrument variation by reference to a barium carbonate-C^{14} standard.

Untreated urine was extracted with 8 volumes of dichloromethane, an aliquot of the dichloromethane extract evaporated *in vacuo*, and the residue dissolved in methanol and plated. The remaining urine was then hydrolyzed with β-glucuronidase. This hydrolysate was then extracted with 8 to 9 volumes of dichloromethane, and an aliquot of the dried dichloromethane residue dissolved in methanol and plated. Aliquots of this extract and of the remaining aqueous residue were then applied to planchets.

An aliquot of the lyophilized feces was digested using the Peters and Gutmann (6) modification of the Van Slyke-Folch (7) procedure for carbon combustion. The carbon dioxide was collected in a solution of barium hydroxide, and titrated with acid. The barium carbonate precipitate was filtered dry on a small circle of filter paper, and this barium carbonate mount placed on a planchet, counted, and corrected to infinite thickness.

The bile samples were applied directly to planchets; a self-absorption correction factor obtained from a self-absorption curve for bile was employed.

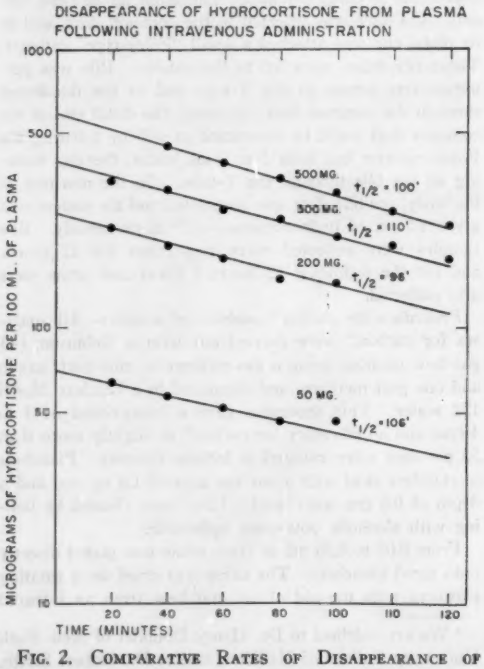


FIG. 2. COMPARATIVE RATES OF DISAPPEARANCE OF HYDROCORTISONE FROM PLASMA FOLLOWING INTRAVENOUS ADMINISTRATION TO A NORMAL SUBJECT

RESULTS

Rate of disappearance of infused steroids from plasma

Rate of disappearance from plasma of hydrocortisone administered intravenously—When plasma hydrocortisone concentrations determined at various time intervals following infusion were plotted on semilogarithmic paper, a straight line was obtained. In Figure 2 are shown the plasma levels and the biological half-times obtained in a single normal subject following the infusion of quantities of hydrocortisone ranging from 50 to 500 mg. The half-times are virtually identical, indicating that the rate of disappearance of hydrocortisone from plasma is proportional to concentration over a wide range of plasma levels. This finding permitted a ready comparison of behavior of infused hydrocortisone in various subjects under various conditions. The biological half-life of hydrocortisone was compared in control subjects and in patients with liver disease, rheumatoid arthritis, hyperthyroidism, hypothyroidism, hypopituitarism, and adrenal cortical insufficiency (Figure 3). In 20 control subjects, representing primarily normal laboratory personnel, the mean biological half-time of hydrocortisone disappearance was 114 ± 6.5 minutes, with a range of 90 to 130 minutes. In 12 patients with mild to moderately severe cirrhosis, both alcoholic and post-hepatitic types, the disappearance of hydrocortisone from plasma was considerably slower, and biological half-times ranging from 160 to 800 minutes were obtained. In some cases markedly abnormal half-times were obtained despite relatively little clinical and laboratory evidence of hepatic dysfunction; however, in all but two cases the diagnosis had been confirmed by liver biopsy. The range of serum bilirubin values in these subjects was 1.6 to 6.0 mg. per cent.

In 8 patients with rheumatoid arthritis, half-time values ranging from 68 to 164 minutes were obtained. Essentially normal values were found in

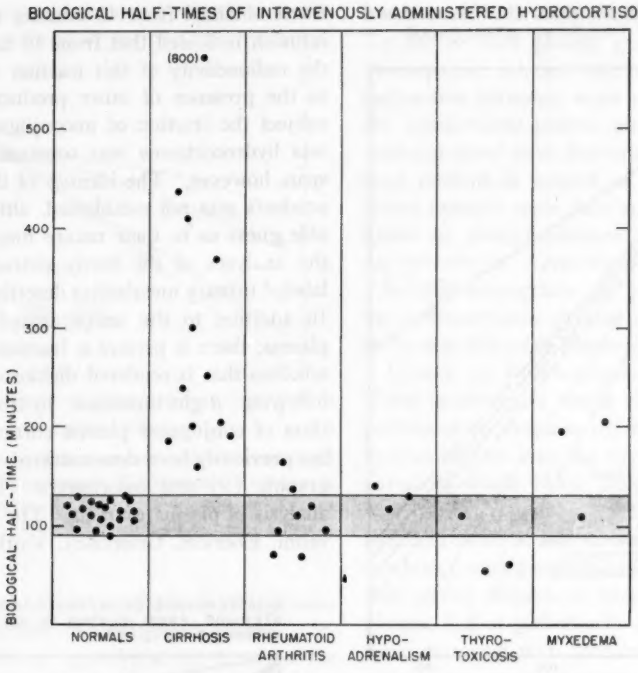


FIG. 3. BIOLOGICAL HALF-TIMES OF INTRAVENOUSLY ADMINISTERED HYDROCORTISONE IN NORMAL SUBJECTS AND IN PATIENTS WITH VARIOUS DISEASES

3 patients with hypoadrenalism. In 3 subjects with hyperthyroidism (BMR in + 25 to + 35 range) somewhat increased rates of hydrocortisone disappearances were found in 2, whereas a normal rate was found in one. In this subject hepatomegaly was present at the time of the study, and later resolved during antithyroid therapy. In 3 subjects with hypothyroidism (BMR in − 40 to − 25 range) delayed rates of hydrocortisone disappearances were observed in 2 and a normal rate in one.

No studies were carried out on patients with hyperadrenalcorticism; however, the rate of disappearance of hydrocortisone from the plasma of a patient with rheumatoid arthritis who had been maintained on 100 mg. of hydrocortisone for 6 weeks, and of another who had been taking 75 mg. a day for more than a year, was within the normal range.

Comparative rates of disappearance from plasma of steroids structurally related to hydrocortisone —Figure 4 demonstrates the rate of disappearance from the plasma in a normal subject of six different steroids [2] structurally related to hydrocortisone, including one with physiologically similar actions (cortisone). Figure 5 demonstrates the disappearance from the plasma of the same steroids in a patient with cirrhosis of the liver. With the exception of hydrocortisone, whose disappearance was greatly delayed in the subject with liver dis-

[2] The cortisone, 11α-hydrocortisone, and tetrahydrocortisone were assayed by the phenylhydrazine procedure as described in the Methods section. The corticosterone, 11α-corticosterone, and 20β-ol-hydrocortisone were assayed by a modification of the fluorometric method of Sweat (3). This method involves exactly the same extraction procedure as described for the phenylhydrazine method. However, the steroid was extracted from the washed dichloromethane extract with one ml. of a reagent containing 7 parts by volume concentrated sulfuric acid and 3 parts by volume redistilled ethanol. The fluorescence of this solution was then determined by using a Farrand Fluorimeter with a primary filter made up of a 470 mμ interference and Corning No. 5113 filter, and a secondary filter containing a 540 mμ interference and Corning No. 3486 filter.

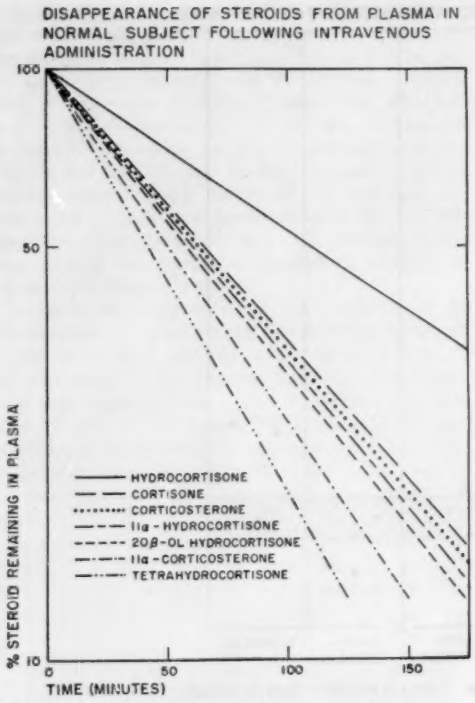


FIG. 4. DISAPPEARANCE OF INTRAVENOUSLY ADMINISTERED STEROIDS FROM PLASMA IN NORMAL SUBJECT

ease, the rate of disappearance of each steroid tested was not strikingly different in the normal and in the cirrhotic subject. Furthermore, all of these steroids disappeared at rates faster than hydrocortisone in both subjects.[3]

Disappearance of radioactive hydrocortisone from plasma—Following intravenous infusion of tracer quantities of hydrocortisone-4-C^{14}, the concentration in plasma of isotopic substances extractable with dichloromethane decreased logarithmically with time (Figure 6). Half-times of 60 to 90 minutes (10 subjects) were obtained. Not all the extracted material represented unaltered hydrocortisone, however, and in 3 subjects the results of chromatographic analyses of plasma extracts obtained from 40 minutes to 4 hours after infusion indicated that from 40 to 60 per cent of the radioactivity of this fraction was attributable to the presence of other products. In a given subject the fraction of unconjugated steroid that was hydrocortisone was constant over this time span, however. The identity of the other labeled products was not established, although a reasonable guess as to their nature may be made from the analyses of the freely extractable group of labeled urinary metabolites described in this paper. In addition to the unconjugated metabolites in plasma, there is present a fraction of labeled metabolites that is rendered dichloromethane soluble following β-glucuronidase hydrolysis. Such a class of conjugated plasma corticoid constituents has previously been demonstrated, using chromatographic (9) and colorimetric (10) methods of analysis of plasma extracts. The data of Bongiovanni, Eberlein, Grumbach, VanWyk, and Clay-

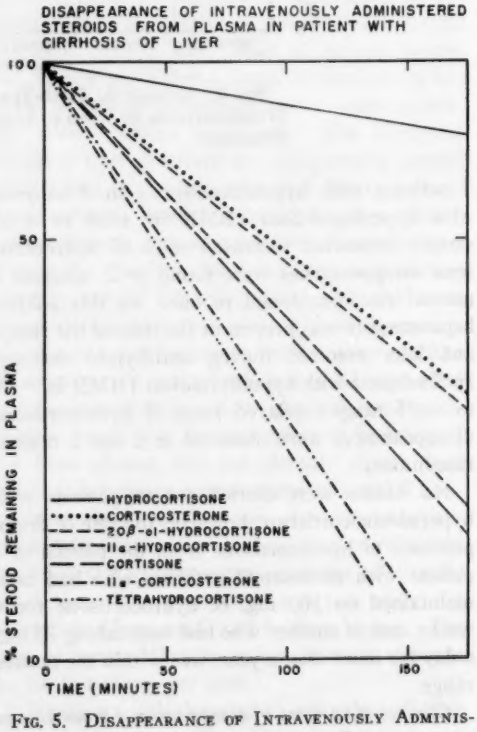


FIG. 5. DISAPPEARANCE OF INTRAVENOUSLY ADMINISTERED STEROIDS FROM PLASMA IN PATIENT WITH CIRRHOSIS

[3] The method described for phenylhydrazine reacting steroids in plasma following their infusion, has been found not to be specific for cortisone (8). Approximately 50 per cent of the material reacting with phenylhydrazine 60 minutes after termination of the infusion is not cortisone. The specificities of the methods used for the other steroids have not been determined.

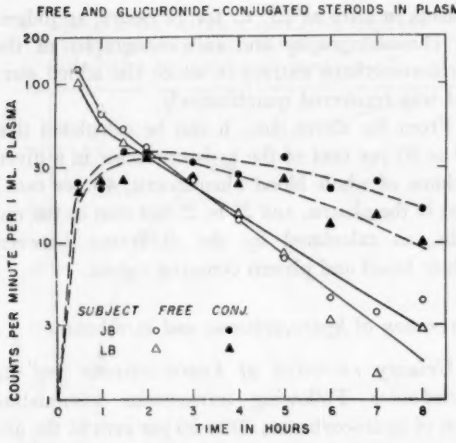


FIG. 6. DIRECT DICHLOROMETHANE EXTRACTABLE ("FREE") AND GLUCURONIDE CONJUGATED RADIOACTIVE STEROIDS IN PLASMA FOLLOWING INFUSION OF TRACER QUANTITIES OF HYDROCORTISONE-4-C^{14} TO NORMAL SUBJECTS

ton (10) suggest that quantitatively the plasma glucuronide fraction of conjugated adrenal hormone metabolites is approximately equal to the concentration of unconjugated phenylhydrazine reactive corticoids which normally consist predominantly of hydrocortisone (11). In the present study, the rate of appearance of labeled metabolites of hydrocortisone in conjugated form in plasma has also been followed (Figure 6). As free steroid disappears from plasma, conjugated derivatives hydrolyzable with β-glucuronidase appear, and reach a maximum at about 2 hours, at which time the free and conjugated fractions are of equal magnitude. Thereafter, the conjugated fraction greatly exceeds the free fraction of labeled steroids. Following extraction of both free and conjugated labeled products, the residual plasma was analyzed for radioactivity, and none was detected. However, self-absorption factors were large so that small quantities of labeled substances may have escaped detection.

Distribution of hydrocortisone in body fluids

Plasma protein binding of hydrocortisone—Fresh human plasma obtained from four normal donors, and isosmotic phosphate buffer of pH 7.4 (0.1 M buffer in M/15 sodium chloride) were equilibrated across a cellophane membrane for 18 hours at 37° C. with gentle continuous motion. To one or both phases, graded amounts of hydrocortisone, labeled with a tracer quantity of radioactive hormone, were added. At the end of the equilibration period, aliquots of both phases were extracted with dichloromethane, and the extract reduced in volume *in vacuo*. The concentrated extracts were then analyzed for hydrocortisone by phenylhydrazine and radioactivity assay procedures. Over the concentration range of 2 to 1,000 micrograms per 100 ml., 75 per cent of the hydrocortisone present in plasma was bound to the non-diffusible elements of plasma.

Uptake of hydrocortisone by erythrocytes—Three *in vitro* and one *in vivo* experiments were performed to evaluate the extent of diffusion of hydrocortisone into the erythrocyte. Fresh heparinized whole blood drawn from one subject was incubated with a tracer quantity of labeled hydrocortisone at 25° C. and 37° C. for one and two hours, with gentle agitation. After the incubation period, aliquots of whole blood, of separated plasma, and of packed cells (hemolyzed in water) were extracted with dichloromethane, and radioactivity measurements made on the extracts. The

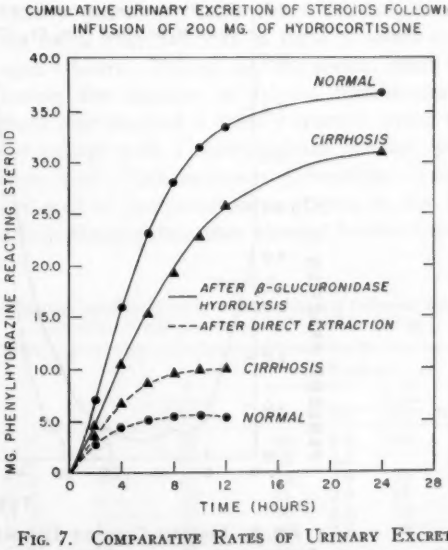


FIG. 7. COMPARATIVE RATES OF URINARY EXCRETION (CUMULATIVE) OF PHENYLHYDRAZINE REACTING STEROIDS FOLLOWING INFUSION OF 200 MG. HYDROCORTISONE TO NORMAL SUBJECT AND PATIENT WITH CIRRHOSIS (ALL ANALYSES MADE ON DICHLOROMETHANE EXTRACTS)

counting values obtained by analysis of the hemolyzed erythrocytes were compared with values calculated for erythrocytes from whole blood and plasma radioactivity concentration data and hematocrit measurements. A similar study was made on whole blood drawn from a normal subject 10 minutes following the intravenous injection of 2.5 μc. of labeled hydrocortisone over a 3-minute period. One fraction of blood was kept at 37° C. for one hour, and another at 25° C. for one hour prior to extraction with dichloromethane. The results of all experiments were similar and indicated that erythrocytes took up hydrocortisone. Solution of hydrocortisone in red cell water appeared to account for only about one-half of the total. Intracellular binding was calculated to account for 49 to 66 per cent of the hydrocortisone present.

Metabolism of the hydrocortisone by whole blood *in vitro* was considered unlikely since varying the time or temperature of incubation did not alter the results. Furthermore, hydrocortisone was not metabolized to a measurable extent in plasma *in vitro* at 25° C. for 72 hours, as judged by chromatography and radioautography of the dichloromethane extract in which the added steroid was recovered quantitatively.

From the above data, it can be calculated that 75 to 80 per cent of the hydrocortisone in a given volume of whole blood (hematocrit, 40 per cent) was in the plasma, and 20 to 25 per cent in the red cells, as calculated by the difference between whole blood and plasma counting values.

Excretion of hydrocortisone and metabolites

Urinary excretion of hydrocortisone and its metabolites—Following intravenous administration of hydrocortisone, 20 to 30 per cent of the administered dose was recovered in the dichloromethane extract of glucuronidase treated urine of the first 24 hours as phenylhydrazine reacting material (Figure 7). An additional 1 to 2 per cent was recovered during the following 24 hours. Approximately one-half of the recovered material appeared in the urine in the first 4 hours. Direct

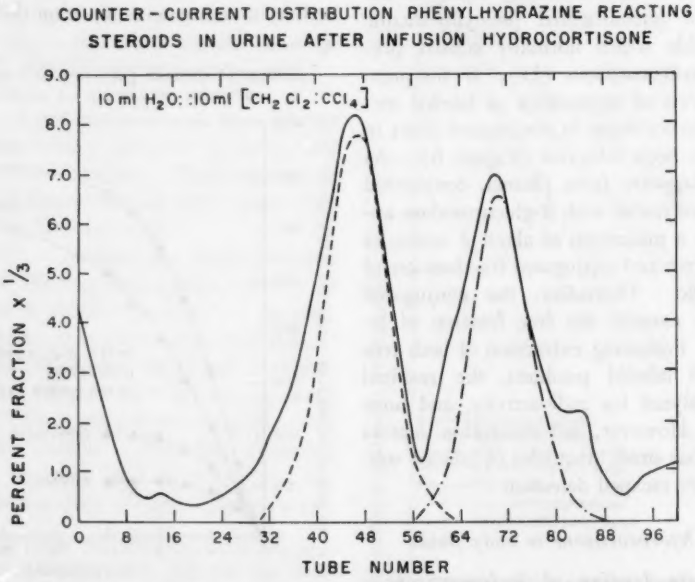


FIG. 8. COUNTER-CURRENT DISTRIBUTION ANALYSIS OF PHENYLHYDRAZINE REACTING MATERIAL IN GLUCURONIDASE HYDROLYZED URINE DICHLOROMETHANE EXTRACT AFTER INFUSION OF 200 MG. HYDROCORTISONE (FIRST 24-HOUR URINE)

—— Experimental curve; – – – Calculated curve of tetrahydrocortisone (K = 0.94); ——·—— Calculated curve of tetrahydrohydrocortisone (K = 3.50).

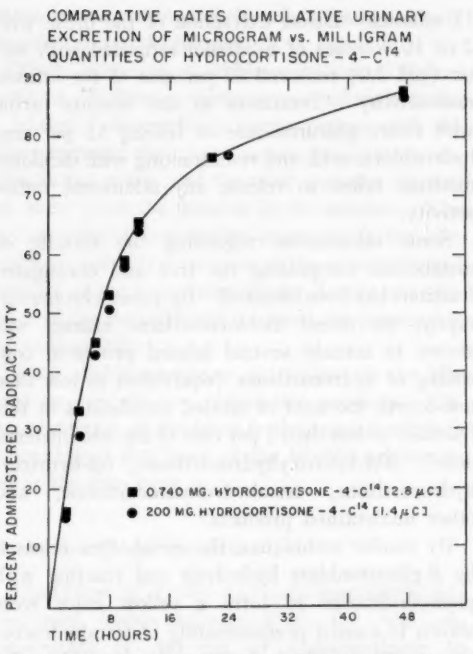

COMPARATIVE RATES CUMULATIVE URINARY EXCRETION OF MICROGRAM vs. MILLIGRAM QUANTITIES OF HYDROCORTISONE - 4-C14
PERCENT ADMINISTERED RADIOACTIVITY
TIME (HOURS)
0.740 MG. HYDROCORTISONE - 4-C14 [2.8 μC]
200 MG. HYDROCORTISONE - 4-C14 [1.4 μC]

FIG. 9. COMPARATIVE RATES OF URINARY EXCRETION (CUMULATIVE) OF RADIOACTIVITY FOLLOWING ADMINISTRATION OF MICROGRAM *vs.* MILLIGRAM QUANTITIES OF HYDROCORTISONE-4-C^{14}

extraction of urine with dichloromethane recovered phenylhydrazine substances equivalent to only 2 to 3 per cent of the administered dose. (Most of the extractable steroid was excreted during the first 4 to 6 hours.) Most of the conjugated metabolites were relatively polar compounds as indicated by a counter-current distribution of a dichloromethane extract of a 24-hour urine in a 99-tube transfer analysis (Figure 8). Two pronounced areas appeared among the polar fractions; however, paper chromatographic fractionation of the material comprising these two areas showed at least two phenylhydrazine reacting compounds in the area on the left and three in the area on the right (more polar). The two components of the area on the left migrated on paper as hydrocortisone and tetrahydrocortisone. One of the compounds in the more polar area on the right migrated as the tetrahydro derivative of hydrocortisone. One of the compounds migrated slightly faster and one slightly slower than the tetrahydro derivative of hydrocortisone. All of these substances (hydrocortisone, tetrahydrocortisone, tetrahydrohydrocortisone) gave the appropriate color reactions with phenylhydrazine and blue tetrazolium, and the anticipated absorption curves in sulfuric acid. In addition, when run as mixed chromatograms, both before and after conversion to the acetates, they behaved in the same manner as the authentic compounds.

Following the infusion of hydrocortisone into a patient with cirrhosis of the liver, the metabolites appeared in urine more slowly than in the normal subject (Figure 7). Also, the total excretion of freely extracted steroid was greater than that found in the normal subject.

Urinary excretion of radiometabolites of hydrocortisone—Figure 9 demonstrates the cumulative urinary excretion of labeled hydrocortisone and metabolites following intravenous administration of a tracer quantity of hydrocortisone-4-C^{14}. Eighty per cent of the injected dose was excreted within the first 24 hours. A small additional quantity appeared during the second and third 24-hour periods, and by the fourth day no labeled urinary products could be demonstrated. In a total of five such studies, an average of more than 90 per cent of the radioactivity was accounted for through urinary excretion (Table II). The half-time of the initial excretion rate of these products averaged 4 hours. During the first several hours following the infusion of labeled hydrocortisone, there was excreted a urinary fraction which was extractable with dichloromethane without prior hydrolysis. This fraction may constitute 15 to 20 per cent of radiometabolites present in the first 2-hour fraction but after about 8 hours virtually

TABLE II

Urinary excretion of carbon^{14} labeled steroid following administration of hydrocortisone-4-C^{14} (200 to 800 μg.)

		% Excreted 24 hrs.	% Total excretion			
			72 hrs.	"Free"	Glucuronide	$t_{\frac{1}{2}}$ hrs.*
L. B.	i.v.	79	92	4.0	60	4.0
J. B.	i.v.	81	89	4.0		4.2
L. B.	i.v.†	80	90	4.0	50	4.0
S. H.	p.o.	82	91	3.0	55	3.5
H. E.	p.o.†	70	76	8.0	45	4.8
R. U.	i.v.	77	89	3.3	60	
R. U.	i.v.	79	94			
W. R.	i.v.	74	86	3.0	50	3.8

* Determined as half-time of the initial rapid excretory rate.

† Plus 200 mg. carrier hydrocortisone.

all products were present in conjugated form. Analysis of a 4-day pool of feces collected following infusion of the steroid revealed an additional 2 to 4 per cent radioactive metabolites of hydrocortisone.

Following intravenous administration of labeled hydrocortisone plus an additional 200 mg. of non-isotopic hydrocortisone, a similar curve of cumulative excretion of hydrocortisone radiometabolites was obtained (Figure 9).

Nature of the hydrocortisone metabolites in the urine—Determination of the nature of the urinary metabolites excreted following intravenous administration of 200 mg. of hydrocortisone labeled with hydrocortisone-4-C^{14} yielded the following results (Figure 10): Direct dichloromethane extraction of the fresh untreated urine recovered 4 per cent of the infused radioactivity, and 3 per cent of the administered steroid as measured with phenylhydrazine. However, following glucuronidase hydrolysis and dichloromethane extraction of the urine, 50 per cent of the administered radioactivity [4] was recovered, but only 17 per cent as measured by phenylhydrazine analysis. An additional 15 per cent of the administered radioactivity could be extracted with dichloromethane following hydrolysis with boiling 15 per cent hydrochloric acid for 15 minutes. Direct extraction of the urine with 2 or 10 volumes of n-butanol saturated with water (pH 2.0) removed 80 per cent of the infused radioactivity. Treatment of the residual urine with either glucuronidase or boiling 15 per cent hydrochloric acid, and re-extracting with dichloromethane failed to release any additional radioactivity.

Some information regarding the identity of metabolites comprising the free and conjugated fractions has been obtained. By paper chromatography, the direct dichloromethane extract was shown to contain several labeled products, consisting of hydrocortisone (equivalent to less than one-fourth the total of labeled metabolites of this fraction, or less than 1 per cent of the administered dose), 20β-hydroxyhydrocortisone, 6β-hydroxyhydrocortisone, tetrahydrohydrocortisone, and other unidentified products.[5]

By similar techniques, the metabolites released by β-glucuronidase hydrolysis and reacting with phenylhydrazine to form a yellow color were shown to consist predominantly of tetrahydrocortisone and tetrahydrohydrocortisone. The corresponding dihydro-derivatives were detected only in small amounts. Evidence for the presence of several additional more polar metabolites was also obtained by radioautography of these chromato-

[4] An additional 2 to 3 per cent more radioactivity can be obtained by reextracting the urine residue with an additional 8 to 9 volumes of dichloromethane. Further treatment of the urine residue with glucuronidase and incubating for an additional 48 hours does not result in the release of an additional amount of radioactivity. Also, treatment of the urine residue with mammalian β-glucuronidase (Viobin 1000 units per ml. urine, or Ketodase 500 units per ml. urine) at pH 4.8, 37° C. for 5 additional days, did not release any more radioactive metabolites extractable into dichloromethane.

[5] These results have been based on a comparison of the rates of migration with the authentic pure compounds, by use of mixed chromatograms, and by conversion of unknown steroids to the acetates and comparing their chromatographic behavior with that of the authentic compounds. These substances also gave the appropriate reactions with phenylhydrazine and with blue tetrazolium, the appropriate sulfuric acid fluorescence results, and the characteristic spectral curves in sulfuric acid.

TABLE III

Plasma levels phenylhydrazine reacting steroid after oral and intramuscular administration of 200 mg. hydrocortisone *

		Hours following administration								
		0	0.5	1	2	3	4	8	12	24
T. R.	p.o.	17	115	220	148	107	91	44	22	15
	i.m.	19		42	57		55	47	37	24
V. L.	p.o.	17		96	160		92	55	18	
	i.m.	16		33	37	30		27	21	15
T. L.	p.o.	25	100	150	190	120	70	25		23
	i.m.	25	26	40	56		56	46	35	28
R. H.	p.o.	13		100	260	255	190			
C. S.	p.o.	16	70	100	140	200	130			
W. R.	p.o.	15	180	260	180		86	18		

* Values are expressed as micrograms per 100 ml. plasma.

grams, but as yet none of these has been positively identified in our laboratory.

Biliary excretion of radiometabolites of hydrocortisone—Following intravenous administration of labeled hydrocortisone to the patient with the biliary cannula, the rate of accumulation of metabolites in the urine, and the total quantity appearing, were much the same as in the normals. The total quantity of metabolites appearing in the bile, however, was only 4 per cent of the injected dose.[6] Analysis of the feces collected for 3 days after infusion of the steroid failed to reveal the presence of any radioactivity.

Direct dichloromethane extraction of the bile failed to recover any radioactive products. Direct extraction of the feces in the normal subjects demonstrated that none of the labeled substances was in the free form.

Absorption of hydrocortisone

Absorption of orally and intramuscularly administered hydrocortisone—With the oral administration of 200 mg. of hydrocortisone to fasting normal subjects, peak plasma levels were obtained in 1 to 2 hours (Table III), indicating rapid absorption of the hormone. The peak plasma steroid concentrations were of the same order of magnitude as the extrapolated initial concentrations obtained following intravenous administration of 200 mg. (Figure 2). The plasma concentration had returned to normal levels within 8 to 12 hours.

Following intramuscular administration of hydrocortisone, peak levels were reached more slowly than following oral administration, and were not nearly as high; however, the concentrations remained elevated for a slightly longer period of time (Table III).

Following oral administration, as with intravenous infusion of hydrocortisone, 20 to 30 per cent of the administered steroid was recovered in the urine as phenylhydrazine reacting metabolites of hydrocortisone. After intramuscular injection an equivalent quantity was recovered; however, it appeared in the urine at a slower rate.

[6] A similar type of experiment revealed an excretion of 4 per cent of administered radioactivity in the bile after infusion of cortisone-4-C^{14}, and 11 per cent after infusion of corticosterone-4-C^{14}.

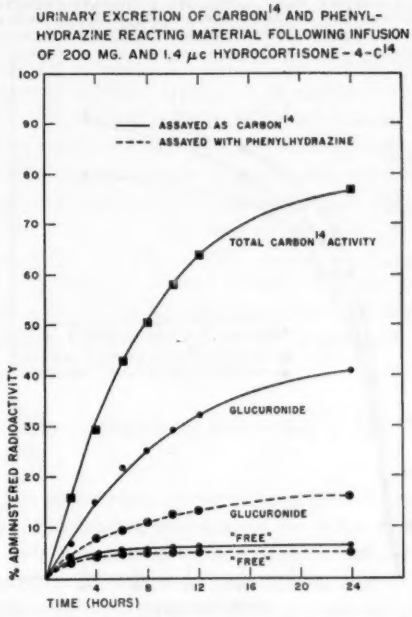


FIG. 10. URINARY EXCRETION (CUMULATIVE) OF RADIOACTIVITY AND PHENYLHYDRAZINE REACTING STEROID FOLLOWING INFUSION OF 1.4 μC HYDROCORTISONE-4-C^{14} CONTAINING 200 MG. CARRIER HYDROCORTISONE

Following oral administration of a tracer quantity of hydrocortisone-4-C^{14}, dissolved in 10 per cent ethanol, a cumulative rate of excretion of metabolites similar to that obtained after intravenous administration was found (Figure 11). Following oral administration of hydrocortisone-4-C^{14} with an additional 200 mg. of carrier steroid in solution in 10 per cent ethanol, a similar pattern of urinary excretion was demonstrated, but slightly less of the radioactivity could be accounted for in the urine. Four per cent appeared in the feces.

DISCUSSION

Disappearance of hydrocortisone from plasma

The rate of disappearance of hydrocortisone from plasma has the characteristics of a first order reaction. Since less than 1 per cent of administered hydrocortisone was excreted as unaltered hormone, the rate of disappearance of hydrocortisone from plasma is presumably also a measure of the rate of its metabolism. The slower disappear-

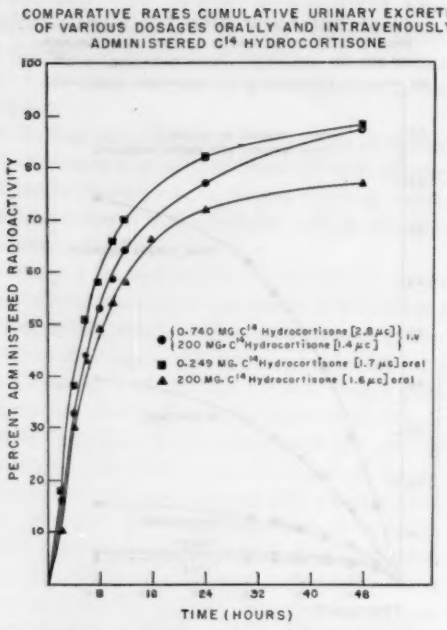

COMPARATIVE RATES CUMULATIVE URINARY EXCRETION OF VARIOUS DOSAGES ORALLY AND INTRAVENOUSLY ADMINISTERED C14 HYDROCORTISONE
PERCENT ADMINISTERED RADIOACTIVITY
TIME (HOURS)
0-740 MG. C14 Hydrocortisone [2.8 μc] } I.V.
200 MG. C14 Hydrocortisone [1.4 μc] }
0-249 MG. C14 Hydrocortisone [1.7 μc] oral
200 MG. C14 Hydrocortisone [1.6 μc] oral

FIG. 11. COMPARATIVE RATES OF URINARY EXCRETION (CUMULATIVE) OF RADIOMETABOLITES FOLLOWING INTRAVENOUS OR ORAL ADMINISTRATION OF HYDROCORTISONE-4-C^{14}

ance of infused hydrocortisone in patients with liver disease, also noted by Brown, Willardson, Samuels, and Tyler (12), is of particular interest. Since this defect is demonstrable in patients with relatively minor liver dysfunction, a test of a subject's ability to handle infused hydrocortisone may serve as a sensitive indicator of hepatic function.

In most cirrhotic patients studied, hydrocortisone was the only steroid for which a striking defect in rate of metabolism was demonstrable. Tomkins and Isselbacher (13, 14) have described a specific triphosphopyridine nucleotide-linked enzyme present in rat liver catalyzing the reduction of the Δ^4 bond of hydrocortisone, but without effect on the Δ^4 bond of other steroids. The product of hydrocortisone reduction is dihydrohydrocortisone, which is then further reduced by a second less specific enzyme (3-hydroxysteroid dehydrogenase [14]) to tetrahydrohydrocortisone. The first reaction is rate controlling, so that the intermediate product does not normally accumulate. Moreover, the dihydro-derivatives do not appear in urine of man to any significant extent (15). These findings suggest that the rate-controlling step in the removal of hydrocortisone from plasma may be the reduction of the A ring of the hormone in the liver, and that it is the enzyme catalyzing this transformation that is deficient in hepatic diseases of the types studied. A finding consistent with this thesis is the observation that tetrahydrocortisone disappears from plasma at a normal rate in patients with liver disease (12) (Figure 5).

The ameliorating effects of jaundice (16) upon active rheumatoid arthritis may well be related to a defect in biological transformation of endogenous hydrocortisone resulting from liver dysfunction. The rate of disappearance of hydrocortisone was clearly delayed in only one of eight rheumatoid arthritics studied.

In two of three patients with thyrotoxicosis, the rate of disappearance of infused hydrocortisone was increased. In two of three with myxedema, hydrocortisone disappearance was delayed. Since similar disturbances were noted after infusion of cortisone, these alterations presumably result from general disturbances of rates of metabolic processes associated with alterations of thyroid function.

In adrenal insufficiency, both primary and secondary, the rates of disappearance of hydrocortisone were normal. Also, in patients who had previously received large doses of hydrocortisone for long periods, the disappearance of infused hydrocortisone was normal. Hellman, Bradlow, Adesman, Fukushima, Kulp, and Gallagher (17) have found the rate of metabolism of hydrocortisone-4-C^{14} normal in adrenal insufficiency as judged by rates and extents of urinary excretion of the labeled metabolites. Apparently hepatic processes which metabolized hydrocortisone proceed without consideration of the peripheral requirements for hormone, being governed by the concentration of hydrocortisone in the circulating plasma, and by the level of enzyme activity in the liver.

Distribution of hydrocortisone in body fluids

The value of the apparent volume of distribution of hydrocortisone depends upon the basis selected for its calculation. Thus, if one utilizes the concentration of hydrocortisone in plasma at zero

time as obtained from an extrapolation of the semilogarithmic plot of plasma hydrocortisone concentrations (Figure 2), considered together with the quantity of hydrocortisone injected, an apparent volume of distribution of 50 to 90 liters can be calculated.[7] (Average = 70 liters [8] in 20 normal and 12 cirrhotic subjects given 50 to 500 mg. of hydrocortisone intravenously.)

In the strictest sense these calculations mean that at the moment of complete mixing it would take a volume of 70 liters to contain all the injected hydrocortisone in the concentration at which it exists in plasma. Since the figures obtained are in all cases close to total body weight, it follows that hydrocortisone is concentrated in some portions of the body at much higher levels than are present in plasma. Tissue localization has been demonstrated to occur in the red cell and in the non-diffusible elements of plasma, and probably occurs in other tissues as well.

When the hydrocortisone space is calculated from the value for the miscible pool as determined by conventional treatment of serial specific activity values of circulating hydrocortisone (18), and the plasma level of phenylhydrazine reacting steroid (largely hydrocortisone [11]), hydrocortisone spaces of 8 to 16 liters (12 subjects) are obtained. The method for determination of the miscible pool depends upon knowledge of the initial concentration of isotopic hydrocortisone in plasma. The addition of new unlabeled hormone will dilute the pool and change its isotope concentration; the removal of hormone from the pool will not. Therefore, only that hydrocortisone capable of mixing with circulating hydrocortisone will be determined by this method.

The discrepancy between values obtained by these two methods is at least in part attributable to the fact that in the estimation of a volume of distribution, equilibration of solute is assumed to have occurred prior to any irreversible transformation or excretion of the test substance. This assumption cannot be valid in the case of hydrocortisone which is known to be metabolized rapidly. The hydrocortisone which is entering such irreversible fates undoubtedly makes a larger contribution to the volume of distribution when measured from hydrocortisone disappearance data than when measured from the isotope dilution data. The value arrived at from isotope dilution data, 8 to 16 liters, is perhaps a fairly accurate indication of the volume of water required to contain the miscible hydrocortisone at the concentration at which it occurs in plasma.

Excretory pathways of metabolites of hydrocortisone

The major route of excretion of labeled metabolites of hydrocortisone was *via* the urine, in which 86 to 94 per cent of the administered carbon^{14} appeared in 72 hours. These figures are in good agreement with those of Hellman and his associates (17) and Plager, Tyler, Hecht, and Samuels (19). Less than one per cent of the administered hormone was excreted as free hydrocortisone. These data suggest that no accumulation of hydrocortisone within the body would result from renal failure such as might be anticipated from hepatic failure.

Only a minor fraction, about 3 per cent, of the administered isotope was recovered in feces. This value is somewhat lower than those reported by Hellman (17) and by Plager and their coworkers (19). The quantity of labeled products appearing in feces was only slightly less than that excreted in bile, suggesting that the bile is the major and perhaps the sole source of fecal products. Furthermore, it appears unlikely that appreciable intestinal reabsorption of biliary metabolites of hydrocortisone occurs. These data are in striking contrast with those obtained in rats and guinea pigs in which 60 to 80 per cent of the administered hydrocortisone-4-C^{14} appeared in biliary metabolites, much of it destined subsequently to participate in an active entero-hepatic circulation in both species (20). These findings are also different from those obtained with progesterone-4-C^{14} in man, where 60 per cent of the radiometabolites appeared in bile (21).

[7] Similar calculations made on the basis of the extrapolated value for the concentration of dichloromethane extractable radioactive substances present in plasma at zero time, after infusion of tracer quantities of hydrocortisone-4-C^{14} can have no significance, since it has been shown that a large fraction of these substances is not hydrocortisone.

[8] This figure of 70 liters is based on the concentrations of free plus plasma protein bound steroid. By employing the free steroid concentration for the calculation, an apparent volume of 280 liters is obtained.

Hellman and his coworkers (17) have reported that less than 0.5 per cent of administered hydrocortisone appeared in the expired air as carbon[14] dioxide. Similar results have been obtained in the guinea pig and rat (20). These findings indicate that the biotransformation of hydrocortisone is not associated with appreciable degradation of the ring structure of the steroid.

Excretion of metabolites in urine

Previous studies have shown that about 20 to 30 per cent of an administered dose of hydrocortisone is excreted as metabolites that are extractable with butanol (22), or become extractable with chloroform or similar solvents following β-glucuronidase hydrolysis (23, 24), and react with phenylhydrazine. Small additional quantities are detectable as 17-ketosteroids, but the sum of all classes of urinary metabolites recognizable by these various methods is less than 40 per cent of the administered dose.

In the present study, recoveries of metabolites in urine, as determined by the phenylhydrazine method after glucuronide hydrolysis agreed well with these earlier reports. However, both in the freely extractable and the glucuronidase hydrolyzed fractions, the percentage of the administered hormone detected by radioactivity analysis was considerably larger than that determined by colorimetric assay. The presence of 20β-hydroxyhydrocortisone as a labeled metabolite of hydrocortisone-C^{14} offers a likely explanation for the discrepancy between results of carbon[14] and phenylhydrazine analyses of the freely extractable fraction since this substance does not react with the phenylhydrazine reagent.

A similar explanation probably applies to the disparity between carbon[14] and phenylhydrazine analyses of the β-glucuronidase-hydrolyzable fraction of conjugated metabolites. Metabolites equivalent to 50 to 60 per cent of administered hormone were released by enzymatic hydrolysis as determined by radioactivity analysis whereas this fraction was 20 to 30 per cent of infused steroid by the phenylhydrazine reaction. Recently, four new urinary metabolites of adrenal cortical hormone have been identified (25). These are derivatives of tetrahydrohydrocortisone and tetrahydrocortisone possessing a 20-ol group in either α or β configuration, apparently present as glucuronides, presumably conjugated at the 3-ol group of each metabolite. These metabolites accounted for about 30 per cent of the neutral extractable steroid products found after the administration of labeled hydrocortisone. These 20-ol derivatives would not react with phenylhydrazine and their presence in urine may well explain the major portion of the difference (equivalent to about 30 per cent of the administered hormone) encountered between the results of the two analytical methods employed in this study.

An additional fraction of 10 to 20 per cent of the infused isotope could be extracted with n-butanol saturated with water, or following hydrolysis with boiling hydrochloric acid followed by dichloromethane extraction. Whether this fraction represents glucuronides resisting hydrolysis with bacterial enzyme, or sulfate or other conjugation products, remains to be determined, as does the nature of the metabolites in the residue completely resisting extraction.

Expressed in terms of the total radioactivity in the urine excreted during the first 24 hours following infusion of hydrocortisone-4-C^{14}, 4 per cent is extracted directly with dichloromethane. An additional 60 to 70 per cent is released following glucuronidase hydrolysis and dichloromethane extraction, and an additional 15 per cent following either n-butanol extraction or dichloromethane extraction after treatment with boiling 15 per cent hydrochloric acid. This accounts for a total of about 85 per cent of the radioactivity present in the urine and leaves approximately 15 per cent unextractable.

Oral absorption

The presence of comparable peak plasma levels after oral and intravenous administration of hydrocortisone, and the similarities of recoveries and rates of excretion of metabolites, suggest that oral absorption of the hormone is both rapid and complete. The fact that the radiometabolites appeared in the urine did not of course exclude the possibility that some of the orally administered steroid was metabolically transformed in the gastrointestinal tract. However, studies carried out on the stability of hydrocortisone-4-C^{14} in fluids from various parts of the gastrointestinal tract

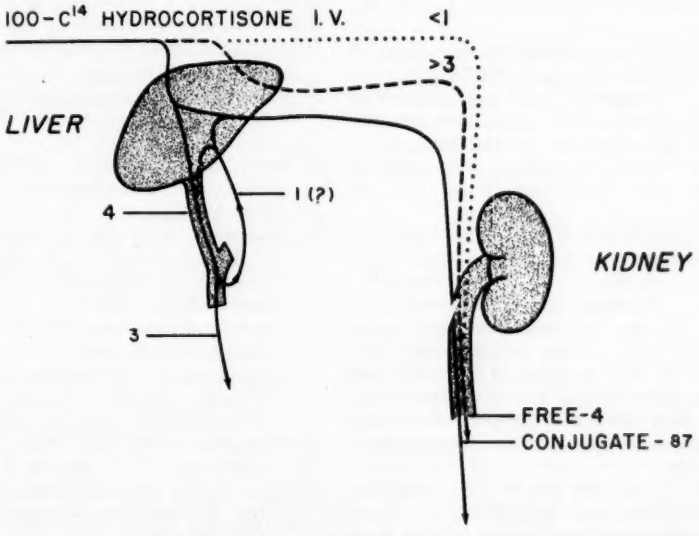
(26) have shown that in gastric juice (pH 1.0) and bile (pH 7.0) no transformation takes place for at least 36 hours (37° C.). In feces, however, after 4 hours all hydrocortisone had been metabolized.

SUMMARY

The physiological disposition and metabolic fate of hydrocortisone have been studied in man following intravenous infusion of hormone in doses up to 500 mg. In normal subjects the half-life of infused hydrocortisone in plasma averaged 1.9 hours. Following the infusion of tracer quantities of hydrocortisone-4-C^{14}, labeled conjugated metabolites appeared in plasma, and at 2 hours their concentration was equal to that of labeled unconjugated substances. Normal subjects excreted over 90 per cent of the administered hormone as urinary metabolites in 72 hours, as judged from excretion of carbon^{14} following infusion of tracer or labeled larger quantities of hormone. Feces of three normal subjects contained 2 to 4 per cent of the administered label, and in one subject with a biliary catheter, 4 per cent of the infused label appeared in the bile. About 4 per cent of the administered hormone was excreted in urine as unconjugated substances; unchanged hydrocortisone represented less than 1 per cent of the administered hormone. The routes of hydrocortisone disposition in man are schematically illustrated in Figure 12. About 60 per cent of the administered hydrocortisone was excreted as metabolites hydrolyzable with bacterial β-glucuronidase, although only about half of this fraction was detectable by the phenylhydrazine reaction.

In patients with liver disease disappearance of infused hydrocortisone was slower than normal. There was an associated increase in the fraction of infused hormone excreted in unconjugated form, and also a slower appearance of conjugated metabolites in urine. The defect in metabolism of hydrocortisone in patients with liver disease was discussed in terms of a low hepatic activity of the specific enzyme catalyzing the reduction of the Δ^4 bond of hydrocortisone.

The rates of disappearance of hydrocortisone



ROUTES OF HYDROCORTISONE DISPOSITION IN MAN

FIG. 12. ROUTES OF DISPOSITION OF HYDROCORTISONE IN MAN AFTER INTRAVENOUS ADMINISTRATION OF "100 UNITS" HYDROCORTISONE-4-C^{14}

.... Urinary excretion of unchanged hydrocortisone; --- Urinary excretion of hydrocortisone metabolized by the liver but not conjugated; —— Urinary and biliary excretion of conjugated metabolites of hydrocortisone.

from plasma were increased in two of three patients with moderately severe thyrotoxicosis, and slowed in two of three with myxedema. Disappearance rates were normal in three subjects with hypoadrenalcorticism.

The absorption of tracer quantities of hydrocortisone from the gastrointestinal tract was shown to be rapid and complete. No destruction of hydrocortisone was detected after incubation of labeled hormone with gastric juice, bile, or serum, but extensive destruction occurred during incubation with feces.

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DISTRIBUTION OF SODIUM BICARBONATE INFUSED INTO NEPHRECTOMIZED DOGS [1]

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The participation of intracellular buffers in stabilizing hydrogen ion concentration in body fluids (1) is suggested by studies in which mineral acid has been administered at rates in excess of renal excretion of acid (2–4). Neutralization as bicarbonate of retained carbon dioxide (5–8) also indicates the role of intracellular buffers when acid loading overwhelms respiratory and renal regulations of acid-base equilibrium. While intracellular buffers might be expected to participate in neutralizing excess alkali, studies of the distribution of administered bicarbonate in cat muscle (9) and in the whole body of man (10) do not allocate to intracellular buffers a significant role in neutralizing administered alkali.

The extent to which blood and tissue buffers participate in neutralizing alkali infused as sodium bicarbonate has been studied by measuring changes in the total quantity of extracellular and erythrocyte sodium, potassium, bicarbonate, and chloride following infusion of sodium bicarbonate into nephrectomized dogs. Preliminary reports (11, 12) were based on experiments in which extracellular fluid volume was approximated by inulin distribution. Subsequent studies in this laboratory indicated that radiosulfate distribution more closely approximates extracellular fluid volume in the whole animal than does inulin distribution (13). Therefore these studies have been repeated using radiosulfate distribution to measure changes in extracellular fluid volume. No definitive publication of the original experiments is contemplated.

The results of our recent experiments indicate that when 20 mM of sodium bicarbonate per kilogram of body weight are infused into a nephrectomized dog over a period of two and a half hours, three-fourths of the added sodium and two-thirds of the added bicarbonate remain in extracellular fluid, while one-fourth of the added sodium probably exchanges for intracellular hydrogen ion.

EXPERIMENTAL PLAN

Dogs were nephrectomized immediately before each experiment to eliminate renal excretion of water and ions and to permit more accurate measurement of the volume of distribution of radiosulfate. Control values for sodium, potassium, bicarbonate, and chloride in extracellular fluid and in the circulating erythrocyte mass were established three and four hours after nephrectomy. One and two hours after the production of severe metabolic alkalosis by infusion of sodium bicarbonate (0.6 normal in the first group of four experiments, 0.3 normal in the second group of four experiments) the total amounts of each of these ions in extracellular fluid and in the erythrocyte mass were re-established. Ion transfers to and from extracellular fluid and into and out of circulating erythrocytes were calculated from the differences in mean ion contents observed in the two control and two alkalosis observations. Results of four control experiments performed under comparable conditions have been reported previously (4).

EXPERIMENTAL PROCEDURE

Healthy, moderately lean, adult male dogs were lightly anesthetized with sodium pentobarbital, weighed and nephrectomized bilaterally. Radiosulfate ($S^{35}O_4$) and (in three of eight experiments) radiochloride (Cl^{36}) were infused as previously described (13). Two and one-half, three, three and one-half and four hours after infusion of radioisotopes arterial blood samples were drawn for measurement of plasma radiosulfate concentration and plasma specific gravity. Additional samples were obtained at three and four hours for measurement of plasma and whole blood sodium, potassium, CO_2, chloride, and for plasma radiochloride concentrations, plasma pH, whole blood water content, hematocrit and, in the second group of experiments, for plasma inorganic phosphate concentration.

Following the four-hour blood sampling sodium bicarbonate was infused through a polyethylene catheter inserted through a femoral vein into the inferior vena cava. In the first group, 0.6 normal sodium bicarbonate was infused at 4 ml. per minute. In the second group 0.3 nor-

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mal sodium bicarbonate was infused at 8 ml. per minute. A total of approximately 20 mM per Kg. of body weight was infused in each experiment. Sodium bicarbonate solutions were equilibrated with 5 to 7 per cent CO_2 before infusion to reduce alkalinity.

One hour following completion of the infusion of sodium bicarbonate and at half-hour intervals thereafter, four arterial blood samples were drawn for re-measurement of all plasma and whole blood components. Plasma volume was measured by T-1824 dilution one and one-half hours before the infusion of sodium bicarbonate was started and one and one-half hours after the infusion was completed.

Approximately 120 ml. of blood were drawn before and a similar amount following the infusion. Autopsy following each experiment verified that renal excretion had been completely eliminated and that hemorrhage at the operative sites had not occurred.

ANALYTICAL METHODS AND CALCULATIONS

The analytical methods used and the methods of calculating results have been described previously (4). The ratio of concentrations of chloride in erythrocyte and plasma water averaged 0.64 in control periods and 0.61 after infusion of sodium bicarbonate.

Since this study was completed the distribution of radiosulfate across cellophane membranes between plasma and an ultrafiltrate of plasma has been studied in this laboratory and indicates a Donnan factor for sulfate of 0.96 (4), rather than 0.90 previously assumed. The measurement of erythrocyte penetration by radiosulfate under comparable experimental conditions indicates an additional small correction should be applied in calculating radiosulfate volumes (14). This latter correction in large part cancels the increase in volume of distribution obtained by using the observed, rather than the assumed, Donnan factor for the sulfate ion. Recalculation of the results of the experiments reported herein using a Donnan factor of 0.96 and correcting for penetration of erythrocytes by radiosulfate does not significantly alter results described below.

RESULTS

In the first group of four experiments the combination of marked alkalosis and marked hypertonicity of body fluids induced by infusing 0.6 normal sodium bicarbonate was poorly tolerated. Varying degrees of tetany, tachypnea, hyperthermia, hypoxia and hypotension developed which may underly the inconstant changes in extracellular ion content observed in this group of experiments as compared to the second group. In the second group of four experiments the infusion of 0.3 normal sodium bicarbonate was well tolerated.

Detailed data from an experiment representative of the first group of experiments are presented in Table I. The infusion of 390 mM of sodium bicarbonate into a 19.9 Kg. dog raised the plasma pH from 7.43 to 7.67, the concentration of sodium in plasma water from 152 mM per liter to 180 mM per liter, the concentration of bicarbonate from 26 mM per liter to 59 mM per liter and reduced the concentrations of potassium and chloride. The increment in the radiosulfate volume exceeds the volume of hypertonic sodium bicarbo-

TABLE I

Average changes in total millimols of each monovalent ion in extracellular fluid and in circulating erythrocyte mass derived from volumes and concentrations measured in an experiment representative of the first group of experiments

Elaps. Time hours	Plasma Water Vol. ml.	$S^{35}O_4$ Vol. ml.	Hemat-ocrit	Plasma pH	Conc. in Plasma Water (millimols per liter) Na	K	HCO_3	Cl	Extracellular (total millimols) Na	K	HCO_3	Cl	Conc. in Erythrocytes (millimols per liter) Na	K	HCO_3	Cl	H_2O %
3.00	900	4355	.430	7.43	151.9	5.1	25.5	113.8	635	21	115	516	103	7.4	10.2	45.4	62.1
4.00			.440	7.43	152.8	5.5	25.5	113.8	640	23	115	516	96	7.3	10.2	46.2	62.0
4.16 - 6.50	Infusion of 650 ml. of 0.596 N $NaHCO_3$ (390 mM), 19.9 Kg. Dog																
7.50	900	5045	.440	7.68	179.0	3.4	59.6	94.8	866	17	314	500	130	6.7	21.3	34.1	60.9
8.50			.435	7.66	180.0	3.4	58.2	95.7	870	16	306	502	129	6.4	20.8	33.8	60.7
Average Change (total millimols)									+230	-5	+195	-15	+22	+0.7	+8	-9	

TABLE II

Average changes in total millimols of each monovalent ion in extracellular fluid and in circulating erythrocyte mass derived from volumes and concentrations measured in an experiment representative of the second group of experiments

Elaps. Time hours	Plasma Water Vol. mL.	$S^{35}O_4$ Vol. mL.	Hemat-ocrit	Plasma pH	Conc. in Plasma Water millimols per liter Na	K	HCO_3	Cl	PO_4	Total Cl mM	Extracellular total millimols Na	K	HCO_3	Cl	Conc. in Erythrocytes millimols per liter Na	K	HCO_3	Cl	H_2O %
3.00	940	4560	.355	7.37	156.7	3.8	20.8	125.0	1.6	750	686	17	99	593	112	7.6	8.9	548	61.1
4.00			.337	7.40	158.2	3.8	20.1	125.1	1.6	771	693	17	95	595	108	7.9	8.6	520	60.6
4.08–6.33	Infusion of 1450 ml. of 0.303 N $NaHCO_3$ (440 mM), 18.9 Kg. Dog																		
7.75	1150	6020	.273	7.63	172.0	2.7	62.0	94.5	2.2	735	993	18	390	594	128	9.7	24.0	37.0	57.9
8.75			.260	7.62	172.5	3.3	62.2	94.7	2.3	737	997	18	391	595	123	8.3	24.4	36.8	58.5
Average change (total millimols)										-24	+305	+1	+294	+1	-3	0	+6	-13	

nate infused. Total extracellular sodium increased 230 mM, bicarbonate increased 195 mM, while extracellular potassium and chloride decreased 5 and 15 mM, respectively. Total sodium and bicarbonate in circulating erythrocytes increased 22 and 8 mM, respectively, while chloride decreased 9 mM.

Detailed data from a representative experiment in the second group are presented in Table II. The infusion of 440 mM of sodium bicarbonate into an 18.9 Kg. dog raised the plasma pH from 7.38 to 7.63, the concentration of sodium in plasma water from 157 to 172 mM per liter, the concentration of bicarbonate from 20 to 62 mM per liter, the concentration of phosphate from 1.6 to 2.3 mM per liter and reduced the concentration of potas-

TABLE III

Summary of data from the eight experiments

Exp. No.	Wt. Kg.	$NaHCO_3$ Infused Total mM	mM/Kg.	Vol. mL.	Δ Total Cl mM	Δ $S^{35}O_4$ Vol. mL.	Final Plasma pH	Δ Conc. in Plasma Water millimols per liter Na	K	HCO_3	Cl	Δ Total Extracellular millimols Na	K	HCO_3	Cl	% Infused Lost From ECF Na	HCO_3
1	18.7	330	17.6	550		+740	7.55	+25	+0.4	+33	-22	+211	+4	+200	-6	26	39
2	26.9	495	18.4	825		+1815	7.60	+27	-0.3	+32	-19	+463	+7	+282	+94	10	43
3	20.9	387	18.5	650		+1213	7.60	+28	+0.8	+37	-21	+309	+8	+218	+42	20	44
4	19.9	390	19.6	650		+690	7.67	+28	-1.9	+33	-19	+230	-5	+195	-15	31	50
Av.	21.6	401	18.5	669		+1114		+27	-0.3	+34	-20	+303	+4	+224	+29	24	44
5	20.5	392	19.1	1305	+4	+1515	7.59	+18	-0.6	+35	-23	+335	+2	+278	+26	15	29
6	17.5	330	18.9	1100	-7	+1225	7.69	+15	-0.7	+38	-28	+250	+2	+231	+5	24	30
7	18.9	440	23.2	1450	-24	+1460	7.63	+15	-0.8	+42	-30	+305	+1	+294	+1	31	33
8	29.3	585	20.0	1950		+1980	7.68	+14	-0.3	+39	-32	+398	+6	+350	+2	32	40
Av.	21.6	437	20.3	1450		+1545		+16	-0.6	+39	-28	+322	+3	+288	+9	26	34

sium and chloride. The increase in radiosulfate volume exceeds slightly the volume of the infusion. Total extracellular sodium increased 305 mM, bicarbonate increased 294 mM, while total extracellular potassium and chloride remained essentially unchanged. Total circulating erythrocyte chloride decreased as erythrocyte bicarbonate increased.

In Table III are summarized the results of the four experiments in each of the two groups. In the first group the four animals, averaging 21.6 Kg., received an average of 18.5 mM of sodium bicarbonate per Kg. of body weight. Extracellular fluid volume, as measured by radiosulfate distribution, averaged initially 20.1 per cent of body weight and increased an average of 1114 ml. following infusion of 669 ml. of approximately 0.6 normal sodium bicarbonate. Total sodium in extracellular fluid increased 303 mM, equivalent to 76 per cent of the sodium infused. Total extracellular potassium increased 4 mM. Total bicarbonate increased 224 mM, equivalent to 56 per cent of infused bicarbonate. The change in total chloride was quite variable, the average increase being equivalent to 7 per cent of the infused bicarbonate.

In the second group of experiments the four animals, averaging 21.6 Kg., received an average of 20.3 mM of sodium bicarbonate per Kg. of body weight. Extracellular fluid volume, averaging initially 22.6 per cent of body weight, increased an average of 1,545 ml. following infusion of 1,450 ml. of approximately 0.3 normal sodium bicarbonate. Total sodium in extracellular fluid increased 322 mM, equivalent to 74 per cent of infused sodium. Total potassium increased 3 mM. Total bicarbonate increased 288 mM, equivalent to 66 per cent of infused bicarbonate, while total chloride increased 9 mM, a change equivalent to only 2 per cent of infused sodium bicarbonate. Total exchangeable chloride (not given in Table III) was 753 mM, 599 mM and 760 mM in experiments 5, 6 and 7, respectively, averaging 37 mM chloride per Kg. of body weight. Total exchangeable chloride increased 4 mM in Experiment 5, decreased 7 mM and 24 mM in Experiments 6 and 7, respectively, decreasing on an average only 1 per cent, a change which is not significant. The average change in total extracellular chloride of 9 mM therefore represents an increase equivalent to only 1 per cent of total body chloride.

In the first group of experiments the change in total sodium, potassium, bicarbonate, and chloride in circulating erythrocytes after sodium bicarbonate infusion averaged + 18 mM (range for the four experiments, + 16 to + 22), 0 mM (+ 0.6 to − 0.7), + 10 mM (+ 8 to + 13) and − 6 mM (− 4 to − 9 mM), respectively. In the second group of experiments these changes averaged − 13 (− 3 to − 20) for sodium, − 1 (− 0.2 to − 2.9) for potassium, + 4 (+ 2.1 to 5.9) for bicarbonate, and − 17 (− 13 to − 24) for chloride. Changes in concentrations of these ions in erythrocytes are consistent in the two groups of experiments as is apparent in Table I and II. The difference in results between the two groups of experiments as regards total ion content of circulating erythrocytes is apparently due to loss of circulating erythrocytes or to underestimation of plasma volume following sodium bicarbonate infusion in the second group of experiments. Reasons for either of these two possibilities are not apparent. Sources of error in these measurements of changes in ion content of circulating erythro-

TABLE IV

Change in osmolarity of plasma water due to monovalent ions and change in anion deficit of plasma water following infusion of sodium bicarbonate

Exp. No.	Milliosmols per liter ($Na+K+HCO_3+Cl$)		Anion Deficit (mEq/L) ($Na+K-HCO_3-Cl$)	
	Before $NaHCO_3$	After $NaHCO_3$	Before $NaHCO_3$	After $NaHCO_3$
1	297.6	342.8	25.0	29.0
2	306.5	347.7	20.9	34.5
3	294.1	339.7	18.9	31.7
4	297.0	337.3	18.4	28.5
Av.	298.8	341.9	20.8	31.1
5	298.2	324.3	14.7	17.4
6	298.1	321.9	16.5	20.2
7	306.3	332.0	15.8	18.6
8	302.0	323.4	18.4	24.3
Av.	301.1	325.4	16.4	20.1

cytes have been discussed by Giebisch, Berger, and Pitts (8).

Table IV summarizes changes in osmolarity of plasma water contributed by the four monovalent ions, sodium, potassium, bicarbonate, and chloride and changes in anion deficit ($Na + K - HCO_3 - Cl$) resulting from the infusion of sodium bicarbonate. Osmolarity increased 43.1 mOsm. per liter in the first group of experiments and 24.3 mOsm. per liter in the second group. The difference between monovalent cations (Na plus K) and monovalent anions (HCO_3 plus Cl) increased 10.3 mEq. per liter and 3.7 mEq. per liter in the first and second group of experiments, respectively.

In the first group of experiments the calculated increase in anion equivalence of plasma protein with rising pH is largely offset by the decrease in concentration of plasma protein following sodium bicarbonate infusion. Anions other than those analyzed for have accumulated apparently in extracellular fluid. In the second group of experiments the decrease in plasma protein concentration following infusion exceeded by 2.3 mEq. per liter the increase in anion equivalence expected from increasing pH. Plasma inorganic phosphate concentration increased an average of 0.4 mEq. per liter in this group of experiments. Thus approximately 5.6 ($3.7 + 2.3 - 0.4$) mEq. per liter of undetermined anion has appeared in plasma following infusion of sodium bicarbonate.

Changes in ion content of circulating erythrocytes and in osmolarity of plasma water contributed by monovalent ions observed in control experiments under comparable conditions have been previously reported (4).

DISCUSSION

These results indicate that in the nephrectomized dog three-fourths of the sodium and two-thirds of the bicarbonate infused as sodium bicarbonate remain in extracellular fluid as measured by radiosulfate distribution. In contrast to earlier reports by us (1) and others (15) of studies based on inulin distribution total extracellular chloride increases only slightly and by an amount approximating the measured decrement in chloride of circulating erythrocytes.

The marked increase in carbonic acid concentration and pCO_2 of plasma which can be calculated from data of Tables I and II represents respiratory regulation of extracellular hydrogen ion concentration. Without this retention of CO_2 hydrogen ion concentration would fall far below limits for survival.

Accumulation of unmeasured anion in extracellular fluid

The fraction of infused bicarbonate disappearing from extracellular fluid exceeds considerably the fraction of infused sodium disappearing from extracellular fluid. From Table IV this apparently results largely from accumulation of unmeasured anion displacing bicarbonate in extracellular fluid to the extent of 5.6 mEq. per liter in the second group of experiments, to a slightly greater extent in the first group of experiments. This anion is probably mostly lactate. Increase in plasma lactate concentration of this magnitude in alkalosis of this degree but of respiratory origin has been observed under comparable experimental conditions by Giebisch, Berger, and Pitts (8) and in metabolic alkalosis by others (16). Regarded earlier as compensatory (17) or the result of tissue hypoxia (18) lactate accumulation may be a more direct effect of alkalosis on carbohydrate metabolism (19).

Fate of fraction of infused sodium leaving extracellular fluid

The fate of the 25 per cent of infused sodium leaving extracellular fluid and of an equivalent amount of bicarbonate is not shown by these experiments. The magnitude of this fraction is in close agreement with that observed by Singer, Clark, Barker, Crosley, and Elkinton (20) despite great difference in degree of alkalosis and in experimental conditions. These investigators infused 2.4 mEq. sodium bicarbonate per Kg. of body weight into normal man over a ten-minute period and observed for one and one-half to two hours changes in concentrations of ions in plasma and change in volume of extracellular fluid as measured by chloride distribution.

When the distribution of sodium bicarbonate in our experiments is calculated on the basis of radiochloride distribution (13), as can be done in the experiments (No. 5, 6, 7) in which total body

TABLE V

Average changes in total millimols of each monovalent ion in extracellular fluid when the volume of the latter is measured by radiochloride distribution *

Exp No	Plasma Vol. Ml.	Chloride Vol. Ml.	RBC Cl mM	$NaHCO_3$ Given mM	Δ Total Extracellular millimols Na	K	HCO_3	Cl	Cl Vol. / $S^{35}O_4$ Vol.
5	1180	5820	30	392	+352	+2	+303	+17	1.14
	1085	7355	17						1.12
6	770	4515	33	330	+301	+2	+271	+12	1.16
	900	6000	13						1.17
7	940	5600	29	440	+402	+1	+408	-21	1.23
	1150	7280	16						1.21
Av.				387	+352	+2	+327	+3	1.18
									1.17

* Where two values are given for each experiment the upper value represents the control period; the value underneath represents the period following sodium bicarbonate infusion.

chloride was measured, a somewhat different distribution is apparent (Table V). The initial ratio of chloride volume to radiosulfate volume is in agreement with that previously calculated (13) and remains unchanged after infusion. Since the chloride volume exceeds the sulfate volume, the fraction of infused sodium and bicarbonate not accounted for in the chloride volume is smaller (9 per cent and 15 per cent, respectively). Reasons for considering that radiosulfate distribution is a better measure of extracellular fluid volume than is chloride distribution have been summarized previously (13).

The fraction of infused sodium bicarbonate not accounted for in extracellular fluid may 1) diffuse into cells, increasing intracellular sodium and bicarbonate accordingly, 2) may be neutralized by intracellular buffers, hydrogen ion derived from cell buffers exchanging with sodium and reacting with bicarbonate to form CO_2 and water or 3) may be neutralized by ion exchange at surfaces of extracellular solid structures (21, 22). These possibilities have been discussed by Singer, Clark, Barker, Crosley, and Elkinton (20).

Regarding possibility 1, Wallace and Hastings (9) calculated that intracellular bicarbonate concentration in muscle remains constant as extracellular bicarbonate concentration increases. The validity of this calculation is in some doubt (23) and from application of Donnan theory to ionic equilibria across cell membranes (24) intracellular bicarbonate concentration would be expected to increase as pCO_2 and extracellular bicarbonate concentration increase.

Possibility 2 and 3 entail loss from body fluids of infused ions. The observed increase in osmolarity of body fluids might be expected to be less than in the case of possibility 1. The expected change in osmolarity of plasma contributed by monovalent ions and change in extracellular fluid volume can be calculated (4) by assuming a) an initial body water of 60 per cent of body weight, b) osmotic equilibrium between cells and extracellular fluid, c) a small insensible water loss of about 75 ml. between the control period and the period of alkalosis and d) that the accumulating unmeasured anion is lactate. The observed increase in osmolarity of plasma water contributed by monovalent ions is less than that expected on the basis of possibility 1, and is in fair agreement with that expected on the basis of possibilities 2 and 3. However, the assumptions are large and the possible error of such calculations too great to distinguish between possibility 2 and 3 or to support the distribution of sodium bicarbonate as revealed by either radiosulfate or radiochloride distribution over that revealed by the distribution of the other.

The implications of the observed distribution of infused sodium bicarbonate in treating metabolic acidosis have been discussed by others (20).

SUMMARY

Sodium bicarbonate, totalling 20 mM per Kg. of body weight, has been infused into nephrectomized dogs and its distribution studied with respect to the measured volume of extracellular fluid. Three-fourths of the infused sodium bicarbonate remains in extracellular fluid. Some of this extracellular fraction is subsequently neutralized by accumulating acid, probably lactic. The remaining one-fourth of the infused sodium bicarbonate leaves extracellular fluid and may be neutralized as sodium exchanges for hydrogen ion derived from intracellular buffers.

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THE ACTION OF IODOACETATE ON THE ELECTRICAL AND MECHANICAL ACTIVITIES OF THE ISOLATED PERFUSED FROG HEART [1]

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The use of enzyme inhibitors on cardiac tissue has permitted a better understanding of the relationship between derangements of metabolism and function. The metabolic and physiologic actions of iodoacetate (IAA) have been reported by a number of investigators (1–4). In low concentrations IAA has been shown to act as a specific inhibitor of triosephosphate dehydrogenase thereby blocking the glycolytic process (5, 6). This is in contrast to its reported action as a general sulfhydryl inhibitor when relatively higher concentrations are employed (6, 7). Studies with C^{14} labeled pyruvate (8) have shown that the addition of 10^{-3} and 10^{-4} M IAA reduces the pyruvate utilization (66 per cent) in heart slices without affecting oxygen consumption whereas greater amounts depress both oxygen consumption and pyruvate disappearance.

Earlier studies on the physiological effects of IAA have demonstrated a depression of contractility and spontaneous rate of isolated rabbit and frog auricles (2, 3). In a recent study on the isolated rabbit auricle Gardner, Wilson, and Farah (4) reported that IAA produced far greater effects on contractility and spontaneous rate than on the excitability or the action potential. They predicated the differences in mechanical and electrical behavior on a difference in the energy requirements of these functions. They believe the IAA-sensitive functions (such as rhythmicity and contractility) require a greater amount of energy than the IAA-insensitive functions (electrical excitability and action potential) and therefore are affected earlier. In further support of this explanation these investigators extended their studies to other enzymes inhibitors such as cyanide, dinitrophenol and fluoroacetate, which showed actions on different stages of the energy-producing processes and differential effects on the various cardiac functions similar to those observed with iodoacetate (9).

The present investigation was designed: 1) To extend the study of the effects of IAA to the electrical activity of single ventricular fibers of the frog's heart; 2) to correlate the electrical with the mechanical events, using cardiac output as an index of the latter; and 3) to observe the modifying effects, if any, of metabolic intermediates such as pyruvate and acetate, and adenosine tri-phosphate (ATP) on the mechanical and electrical activities of the IAA-treated heart.

METHODS

Frogs (*Rana pipiens*) in winter stage were used. The animals were pithed and the perfusion technique as described by Bülbring (10) was employed. Frog Ringer's solution was the perfusion medium and contained the following in mEq. per L.: NaCl 110; KCl 4.02; $CaCl_2$ 1.53; $NaHCO_3$ 4.29; and glucose 4.11. The venous pressure was varied between 2 and 2.5 cm. H_2O and the aortic resistance was fixed at a level of 3 cm. above the heart. Iodoacetate (5×10^{-3}M) was administered as a constant perfusion. The intracellular action potential was recorded simultaneously with an indirect electrocardiogram (Lead I) on a Sanborn Twin Beam Model 62 electrocardiograph. For the technical details of recording the intracellular potentials reference may be made to a previous publication (11). Cardiac outputs were obtained prior to and at frequent intervals after the administration of the antimetabolite.

In the experiments employing the substrates pyruvate (0.0055M) and acetate (0.0055M), and ATP (1.0×10^{-4}M), these agents were added to the IAA-Ringer's solution and given as a continuous perfusion.

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RESULTS

Iodoacetate (5×10^{-5}M) modified both the electrical and mechanical activity of the isolated perfused frog's heart. The effects on both activities were of similar degree and occurred usually at the same time (Figure 1). The most consistent early change in the electrical record was a shortening of the action potential duration (APd) which was progressive. Coincident with this, a shortened S-T interval and an abnormal T wave were observed in the indirect electrocardiogram. Of lesser frequency, and occurring relatively late, was a prolongation of the P-R interval. During the course of the experiment, the height of the membrane action potential (MAP) slowly decreased with impaired depolarization occurring as a relatively late phenomenon. The changes in the mechanical activity were represented by a progressive decrease in cardiac output and stroke volume. Shortly after 120 minutes, the cardiac output dropped to less than 0.1 ml. and in some instances to zero and the magnitude of the membrane resting and action potentials decreased rapidly. When ventricular arrest occurred in from one to several hours atrial activity still persisted for 10 to 20 minutes. The alterations in the repolarization phase of the ventricular fiber were usually paralleled by a change in cardiac output. Figures 1 and 2 are representative of the changes described, and a summary of the data in 14 experiments is shown in Table I.

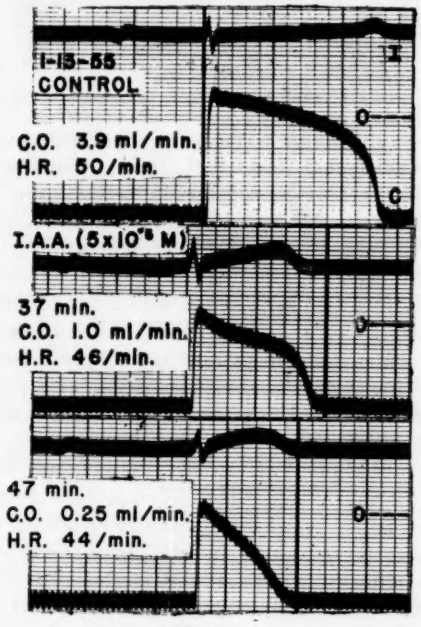

FIG. 1. THE EFFECT OF 5×10^{-5}M IODOACETATE (IAA), ADMINISTERED AS A PERFUSION, ON THE ELECTRICAL AND MECHANICAL ACTIVITIES OF THE ISOLATED FROG HEART

See text for discussion. Simultaneous records are of the indirect electrocardiogram (I) and the single fiber membrane action potential (C). Cardiac output (C.O.) and heart rate (H.R.) were taken just prior to recording of the electrical potentials. Ordinates-millivolts; abscissae-time. Time lines, 0.04 sec. Gain, 0.9 cm. = 50 mv. for intracellular potential; for indirect electrocardiogram (I) 1.0 cm. = 1 mv.

All measurements were made from the top of the action potential trace. Dotted line indicates zero level of potential (O) for the membrane.

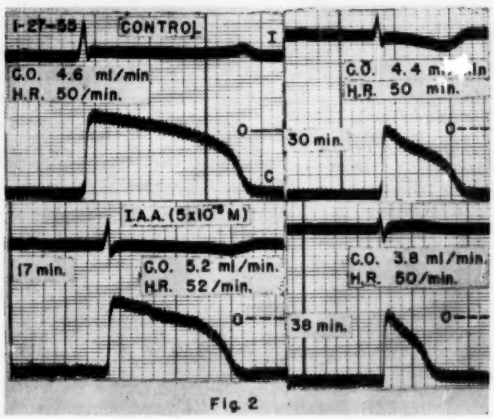

FIG. 2. PROCEDURES SAME AS DESCRIBED IN FIGURE 1.

The addition of the substrates, pyruvate (0.0055M) and acetate (0.0055M) to the IAA treated heart produced only a partial recovery and delay of ultimate deterioration of cardiac activity (Figure 3). The results were similar when ATP (1.0×10^{-4}M) was administered (Figure 4). Doubling the concentration of these substances did not alter the results significantly.

DISCUSSION

In the present investigation, low concentrations of IAA (5×10^{-5}M) were used in order to study its action as a specific inhibitor of triosephosphate dehydrogenase (5, 6) rather than as a general

TABLE I

Action of iodoacetate on the frog heart *

(Averages of 14 experiments)

Time (*minutes*)	Rhythmicity H. R. *per min.*	Contractility C. O. *ml./min.*	Contractility S. V. *ml.*	Indirect bipolar electrocardiogram P-R *sec.*	Indirect bipolar electrocardiogram QRS *sec.*	Indirect bipolar electrocardiogram Q-T *sec.*	Action potential MAP *mv.*	Action potential MAPD *sec.*	Action potential APd *sec.*	Action potential MRP *mv.*	Action potential Overshoot *mv.*
Control	47	3.0	0.064	0.279	0.047	0.588	117	0.017	0.604	95	22
0–20	48	3.0	0.062	0.275	0.045	0.585	116	0.017	0.590	94	22
20–40	50	2.0	0.040	0.270	0.037	0.359	113	0.009	0.352	96	17
40–60	50	1.1	0.022	0.293	0.037	0.308	105	0.011	0.308	93	12
80–100	54	0.9	0.011	0.411	0.040	0.339	101	0.029	0.329	81	20
100–120	52	0.2	0.004	0.357	0.041	0.243	103	0.028	0.250	91	12

* H. R. —Heart rate.
C. O. —Cardiac output.
S. V. —Stroke volume.
MAP —Membrane action potential.
MAPD—Duration of depolarization of membrane action potential.
APd —Duration of action potential.
MRP —Membrane resting potential.

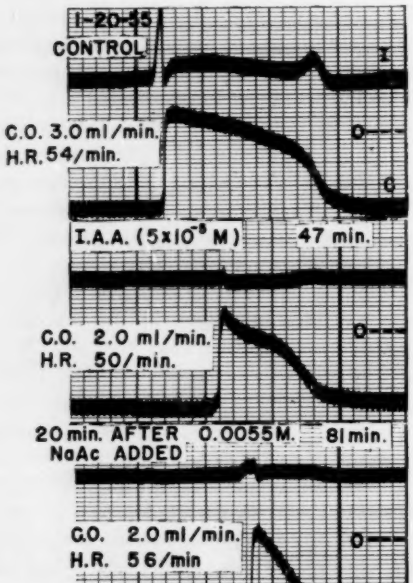

Fig. 3

FIG. 3. THE EFFECT OF 0.0055M SODIUM ACETATE (NA AC) ADDED TO THE IAA PERFUSED FROG HEART

See text for discussion. Indirect electrocardiogram (I, above); membrane action potential (C, below). Cardiac output (C.O.) and heart rate (H.R.) were taken immediately preceding the electrical recordings. Time lines, gains and zero of potential as in Figure 1.

sulfhydryl inhibitor which is the case with concentrations in excess of 10^{-3}M (6, 7). The observations were extended over a relatively long period (1 to 3 hours) in contrast to some of the earlier experiments (2). The most striking finding in this series was the similar degree of susceptibility of the electrical and mechanical properties to the action of IAA (Table I). The alterations in mechanical activity were usually parallelled by changes in the electrical potential, the most prominent of which was enhanced repolarization of the cell (shortened APd). This is at variance with the observations of Gardner, Wilson, and Farah (4) stated above. The different cardiac tissue and dosages employed by Gardner, Wilson, and Farah (4, 9) do not, in our opinion, account for the differences in the results obtained. It is believed that the diverse results are ascribable to the different techniques employed for recording the action potential. Gardner, Wilson, and Farah (4, 9) measured only its height. Detailed analysis was difficult because of the variations observed from auricle to auricle. Reduction in the height of the action potential was reported as gradual, but considerably less than the percentage reduction in contractility. By means of the intracellular microelectrode utilized in our study, it was possible to measure all the parameters of the intracellular action potential and the results disclosed a close correlation and parallellism between electrical and mechanical events.

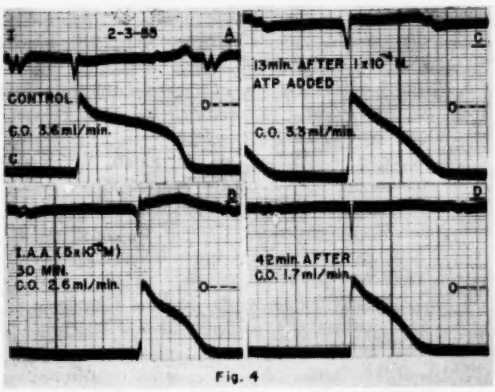

FIG. 4. THE EFFECT OF 1.0×10^{-4}M ADENOSINE TRIPHOSPHATE (ATP) ADDED TO THE IAA PERFUSED FROG HEART

Discussion in text. Indirect electrocardiogram (I, above); membrane action potential (C, below). Technique and symbols are as indicated in Figure 1.

Although it is probable that differential energy gradients exist for the various cardiac functions, it is difficult to explain the changes observed on the basis of quantitative differences in energy requirements. The fact that the repolarization phase of the action potential was influenced at approximately the same time as the cardiac output and stroke volume and at times preceded the latter argues strongly against Gardner, Wilson, and Farah's explanation. It is possible that under the conditions of our experiments, IAA does not act solely on energy pathways and the early modifications of the electrical potential may be due to a direct effect on the membrane *per se*. It is significant that Reilly, Riker, Whitehouse, and Kuriaki (12), using methyl fluoroacetate (MFA) on the papillary muscle of the cat, also observed that those events associated with the electrical restorative processes of the fiber are profoundly altered, as are the effects on the contractile mechanism. The continued interference with repolarization by MFA led to a decay in the contractile mechanism and eventually to conductive failure. Of added interest was the observation that stronger antioxidative agents like cyanide, disrupted both the mechanical and electrical events simultaneously and quite rapidly. The experiments of Reilly, Riker, Whitehouse, and Kuriaki (12) and our own, using MFA and IAA respectively, indicate that alterations of repolarization are usually associated with a change in contractility. To demonstrate this it is essential to measure all components of the electrical and mechanical phases of cardiac activity.

Our results with the substrates, pyruvate and acetate, are contrary to those of Chang (2) who reported that pyruvate and other metabolic intermediates completely reversed the effects on contractility and spontaneous rate caused by iodoacetate. On the other hand, Gardner, Wilson, and Farah (4) observed that recovery by these substrates could be demonstrated only with short exposures to iodoacetate and even then were not as striking as shown by Chang. With longer exposures, as was employed in our experiment, the metabolic intermediates were only briefly effective. The inability to counteract the IAA by these substrates suggests that an additional site of action of the inhibitor exists. The latter opinion has also been expressed by other investigators (9, 13). It has been claimed that in relatively large doses, IAA acts as a general sulfhydryl inhibitor, whereas in low concentrations in the isolated enzyme system it has been shown to be a specific inhibitor of triosephosphate dehydrogenase. It is pertinent that Reisberg (14), studying the effect of various sulfhydryl inhibitors on the action of cholinacetylase reported no impairment with IAA in concentrations of 5×10^{-5}M or less.

The exact mechanism responsible for the enhanced repolarization phase of the trans-membrane potential observed with IAA is not known. If we accept the postulate of Hodgkin (15) that sodium permeability is principally associated with the phase of depolarization and potassium permeability with the phase of repolarization of the action potential, it is suggested that the action of IAA is associated with an enhanced permeability to potassium during cellular activity. The recent studies of Love, Cronvich, and Burch (16) on the mechanism controlling cation concentrations in the human erythrocyte revealed that iodoacetate was associated with an increased outflow of cellular potassium and a net gain of sodium. The observations lend support to our interpretation.

SUMMARY AND CONCLUSIONS

Microelectrodes were inserted into the interior of single ventricular fibers of isolated perfused

frog hearts, and the effects of 5×10^{-5}M iodoacetate on the intracellular potential, the indirect electrocardiogram and cardiac output were recorded.

Prominent changes in both the electrical and mechanical properties were observed. The most consistent and earliest of these was a shortening of the duration of the action potential (APd) and this was progressive. Coincident with this a shortened S-T interval and abnormal T wave were observed in the indirect electrocardiogram. Of lesser frequency and occurring late was a prolongation of the P-R interval. The height of the MAP slowly decreased with impaired depolarization occurring as a relatively late phenomenon. The changes in mechanical activity were represented by a progressive decrease in cardiac output and stroke volume. Auricular activity persisted for 10 to 20 minutes after ventricular arrest. The early alterations in mechanical activity of the ventricle were usually parallelled by changes in the repolarization phase of the action potential (shortened APd). The observation makes it difficult to ascribe quantitative differences in energy requirements to these two cardiac functions.

The addition of substrates, pyruvate (0.0055M) and acetate (0.0055M), to the IAA perfused heart produced only a partial recovery and delay of ultimate deterioration of cardiac activity. A similar result was obtained when adenosine tri-phosphate (1.0×10^{-4}M) was added. Doubling the concentration of these substances did not significantly alter the results. The inability of these metabolic intermediates to restore the cardiac activity suggests that IAA has actions other than specific inhibition of triosephosphate dehydrogenase.

It is postulated that the enhanced repolarization is associated with an increased migration of K^+ out of the cell during its electrical activity.

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INACTIVATION OF THROMBOPLASTIN IN HUMAN BLOOD

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In the course of blood clotting, a potent thromboplastic activity develops, which is no longer present in serum. Biggs, Douglas, and Macfarlane (1) in early studies with the thromboplastin generation test found that prolonged incubation of the generating mixture was accompanied by loss of thromboplastic activity. These investigators suggested that this loss could result either from intrinsic instability of blood thromboplastin or from a neutralization effect. Similar results were obtained by Spurling and King (2), who demonstrated progressive loss of thromboplastin on prolonged incubation when serum was used as the source of plasma thromboplastin component (PTC) in the thromboplastin generation test.

The data to be presented suggest that the thromboplastin formed by blood components is inactivated by a plasma protein contained in the crude albumin fraction.

METHODS AND MATERIALS

Human and bovine plasmas were obtained from blood to which had been added one-tenth volume of 0.1 molar potassium oxalate.

Barium sulfate adsorbed plasma was prepared as follows: For each cc. of plasma 0.1 cc. of molar $BaCl_2$ was mixed with 0.1 cc. of Na_2SO_4. The barium sulfate precipitate was washed three times in isotonic saline. The plasma was mixed with the washed barium sulfate, and adsorption allowed to proceed for ten minutes at about 20° C. The barium sulfate was finally removed by centrifugation.

Human serum was obtained from blood defibrinated with glass beads as described by Biggs and Douglas (3).

Platelet extract was made as follows: A silicone technique was employed throughout. Two liters of oxalated bovine blood were centrifuged for 20 minutes at 1,200 rpm in a Universal PR-1 refrigerated centrifuge kept at 20° C. The platelet-rich supernatant plasma was then separated, and the platelets were sedimented by centrifugation for one hour at 3,000 rpm. The platelets were pooled, and were subjected to three successive suspensions and sedimentations in changes of isotonic saline. The platelet mass finally obtained was homogenized into 25 cc. of isotonic saline, and diluted to 200 cc. with distilled water. This suspension was frozen and thawed twice, and then further homogenized in a Waring Blendor. The particulate matter was removed by centrifugation at 3,000 rpm for 10 minutes, leaving a clear supernatant fluid. This supernatant was frozen at minus 20° C. in 2 cc. aliquots which were thawed as needed.

All coagulation procedures were performed in a water bath at 37° C.

Recalcification of clotting mixtures was accomplished with 0.025 molar calcium chloride.

The thromboplastin generation test was derived from the method of Biggs and Douglas (3), and was performed as follows: Fifteen hundredths cc. each of platelet extract, barium sulfate adsorbed plasma, and serum were mixed, and allowed to incubate for one minute, at which point 0.15 cc. of $CaCl_2$ was added. This mixture was then assayed for thromboplastic activity at designated intervals after recalcification. This assay of thromboplastic activity was accomplished by adding 0.1 cc. of the mixture to 0.1 cc. of untreated bovine plasma which had been recalcified exactly 15 seconds previously with 0.1 cc. of $CaCl_2$.

RESULTS

Preliminary studies were undertaken to determine whether the loss of thromboplastic activity in the thromboplastin generation test was due to instability of the thromboplastin itself or whether it resulted from the inactivating effect of some plasma components. To distinguish between these possibilities, the behavior of the generating mixture was studied with two concentrations of reacting ingredients. In one the barium sulfate plasma and serum were used undiluted; in the other the serum was diluted to 10 per cent and the plasma to 20 per cent with isotonic saline. The concentration of the platelet extract was held constant. In each case serial determinations of thromboplastic activity were obtained for over an hour. The results of these studies are shown in Figure 1. Both mixtures attained a comparable degree of thromboplastic activity after four minutes of incubation. However, it is evident that the mixture containing the undiluted ingredients showed

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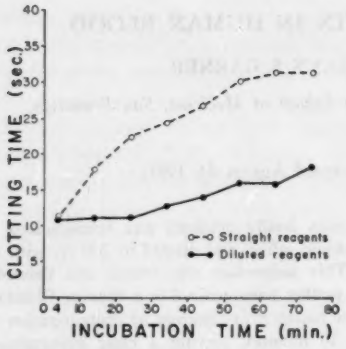

FIG. 1. EFFECT OF REAGENT CONCENTRATION ON THE LOSS OF THROMBOPLASTIC ACTIVITY IN THE THROMBOPLASTIN GENERATION TEST

a more rapid and more profound loss of thromboplastic activity after further incubation. These findings indicate that the loss of thromboplastic activity was due to inactivation by some component found in blood. Accordingly, an assay method was devised to measure the ability of a specimen to inactivate blood thromboplastic activity.

The thromboplastin inactivation test

After a series of preliminary investigations, the following procedure was found to give the most reproducible results: The basic steps are similar to those described for the thromboplastin generation test, with a few additions and modifications. The initial incubating mixture consists of equal volumes of 10 per cent barium sulfate beef plasma, 10 per cent human serum (both diluted with isotonic saline), and platelet extract. One minute after these reagents are mixed an additional volume of $CaCl_2$ solution is added. Four minutes after recalcification the mixture is assayed for thromboplastic activity as above. At five minutes further thromboplastin generation is stopped by decalcification and the test specimen is added to the generating mixture. This test specimen consists of 3 volumes of the unknown solution mixed with 1 volume of 0.025 molar sodium citrate. One volume of test specimen is added to one volume of generating mixture. One minute, and 30 minutes after the addition of the test specimen the mixture is tested for thromboplastic activity. Each time a series of thromboplastin inactivation tests is run, a blank is substituted for the test specimen in the five-minute addition. This blank consists of citrated saline instead of unknown solution, and serves as a check on loss of thromboplastic activity from reactions in the original generating mixture. The essential steps in the thromboplastin inactivation test are shown diagrammatically in Figure 2.

The thromboplastin inactivation test was performed on sera obtained from healthy volunteers among the hospital staff and medical students. Figure 3 summarizes the results obtained. There is slight loss of thromboplastic activity in the saline blank, which results partly from dilution, and probably from the action of the initial reagents as well. The normal sera show a strikingly increased ability over the saline control to elicit thromboplastin inactivation. This inactivation is progressive, increasing with greater duration of incubation. As judged by dilution curves of the original generating mixture (3), more than 90 per cent of the thromboplastic activity formed is inactivated after thirty minutes of incubation with serum. The curve obtained when serial dilutions of serum are used in the thromboplastin inactivation assay is shown in Figure 4. The amount of thromboplastin inactivated appears to be roughly proportional to the concentration of serum. Oxalated plasma has the same ability to inactivate thromboplastin as has serum.

Properties of the thromboplastin inactivator

Preliminary manipulation of serum showed that the thromboplastin inactivator is stable to storage

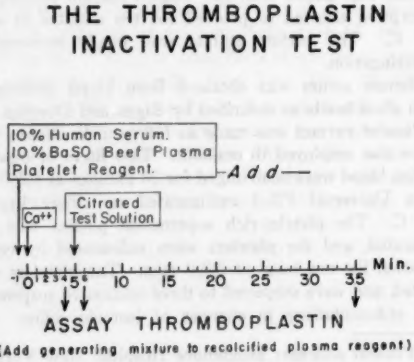

FIGURE 2

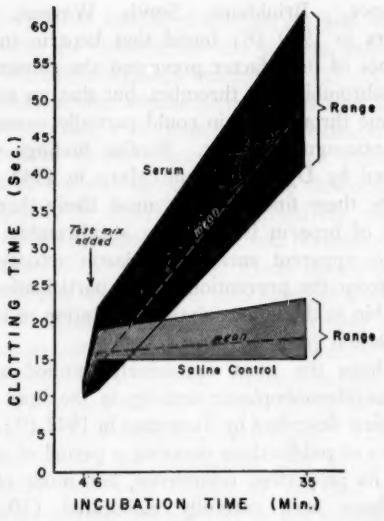

FIG. 3. THROMBOPLASTIN INACTIVATION BY NORMAL HUMAN SERA AS COMPARED TO SALINE CONTROLS

both at room and refrigerator temperatures for at least one week. It was present undiminished in serum stored for over one month at − 20° C., and in plasma that had been lyophilized. It withstood heating at 56° C. for 30 minutes in serum, but was partially destroyed at a temperature of 60° C. after 10 minutes of incubation. It was not removed from serum by barium sulfate or kaolin, nor could it be extracted with repeated ethyl ether treatment. The thromboplastin inactivator was not removed from serum by dialysis against isotonic saline.

Commercially prepared Cohn fractions of human plasma were obtained in dried, powdered form.[2] These fractions were dissolved in isotonic saline so that the protein concentration of each was approximately equivalent to its normal physiological level. The solutions of the various fractions were then tested for thromboplastin inactivator activity. As seen in Figure 5, only fraction V had significant activity, and this activity appeared to represent a quantitative yield of the original plasma content. Full activity was present in outdated lots of liquid Red Cross salt-free albumin stored at refrigerator temperature, and was found in several different lots of "crude" fraction V. Two lots of purified fraction V were also tested. In these preparations, certain trace proteins of fraction VI had been removed, leaving almost pure albumin. Although the protein content of these solutions was the same as that used in testing crude fraction V, the purified fractions were inert with respect to thromboplastin inactivator. These data indicate that the thromboplastin inactivation effect is not the result of a nonspecific protein reaction. Moreover, the thromboplastin inactivator is evidently not albumin *per se*. This view is further confirmed by a study of sera from two patients suffering from the nephrotic syndrome. Despite marked reduction of albumin in these sera, as shown by paper electrophoresis, both had normal thromboplastin inactivator activity.

An attempt was made to separate thromboplastin inactivator from albumin by electrophoresis convection (4). A 4 per cent solution of fraction V was prepared in a citric acid-phosphate buffer solution of 0.1 ionic strength and pH 4.8. The run was performed at room temperature for 24 hours at 0.9 millivolt in a Beckman electrophoresis convection apparatus. Partial purification of the albumin resulted from this single run, with the majority of alpha$_1$ globulin concentrated in the upper chamber of the apparatus. The throm-

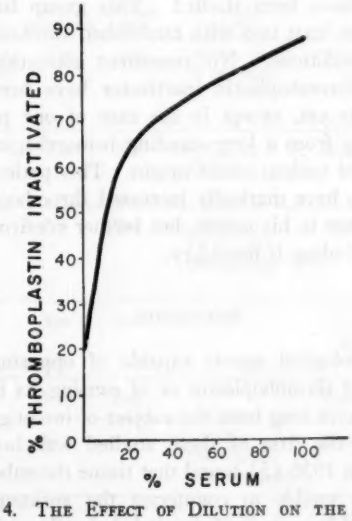

FIG. 4. THE EFFECT OF DILUTION ON THE ABILITY OF NORMAL HUMAN SERUM TO INACTIVATE THROMBOPLASTIN

[2] Kindly provided by Dr. Fred Johnson of the Cutter Laboratories, Berkeley, Calif.

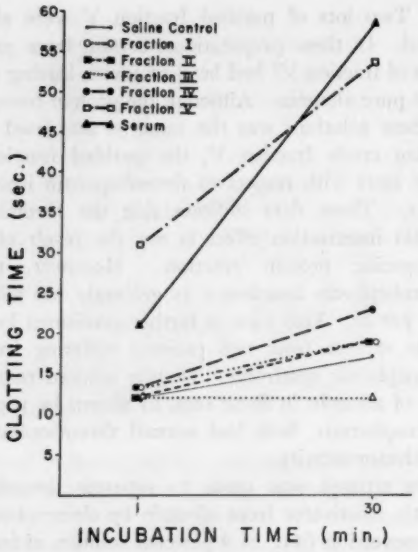

FIG. 5. THROMBOPLASTIN INACTIVATION BY FRACTIONS OF NORMAL HUMAN PLASMA

boplastin inactivator activity was found in the bottom chamber together with the albumin.

Thromboplastin inactivator in disease

To date about 20 patients with a variety of disorders have been studied. This group has included at least two with established thrombo-embolic conditions. No consistent alterations of serum thromboplastin inactivator have been detected as yet, except in the case of one patient suffering from a long-standing hemorrhagic syndrome of undetermined origin. This patient appears to have markedly increased thromboplastin inactivator in his serum, but further confirmation of this finding is necessary.

DISCUSSION

Physiological agents capable of opposing the action of thromboplastin or of causing its inactivation have long been the subject of investigation. Among the first of these studied was heparin. Quick in 1936 (5) found that tissue thromboplastin was unable to counteract the anticoagulant action of heparin, and concluded that heparin did not exert its major action as an antithromboplastic substance. Brinkhous, Smith, Warner, and Seegers in 1939 (6) found that heparin in the presence of its cofactor prevented the conversion of prothrombin into thrombin, but that an excess of tissue thromboplastin could partially overcome this anticoagulant effect. Similar findings were obtained by Dyckerhoff and Marx in 1944 (7). Despite these findings, it is most likely that the action of heparin is primarily antithrombic, and that its apparent antithromboplastin activity results from the prevention of the participation of thrombin in the autocatalytic acceleration of blood coagulation (8).

Perhaps the most extensively studied agent with antithromboplastic activity is the lipid material first described by Tocantins in 1942 (9). In a series of publications covering a period of many years its properties, occurrence, and mode of action have been carefully elaborated (10–18). Since this lipid antithromboplastin is thought to act against cephalin, it has been termed "anticephalin." Anticephalin is present in blood carefully collected in non-wetting containers, and is widely distributed in almost all tissues. It is readily adsorbed on glass and other surface-active materials, and can be concentrated and purified by successive methanol and ether extraction. Anticephalin appears to react directly with thromboplastins of tissue origin and with cephalin, reducing their ability to accelerate clotting in recalcified plasma. According to Tocantins, anticephalin is one of the factors preserving the fluidity of the blood by preventing coagulation in the presence of weak thromboplastic activity. Its action can be overcome by dilution of blood, since it is more susceptible to dilution than are the coagulant substances. It can likewise be made ineffective by contact with an adsorbing surface or overwhelmed by an excess of thromboplastin. A similar substance was described by Overman (19) who concluded that it acted in combination with a protein to form thromboplastin, but that it was anticoagulant if present in excess.

Several groups of investigators have been concerned with plasma proteins capable of inactivating tissue thromboplastin. Schneider (20) was able to identify as thromboplastin a placental toxin which was lethal to mice by the intravenous route. He noted further that this placental thromboplastin gave reduced toxicity and thromboplastic

activity when incubated with serum, and was able partially to characterize the protein responsible for this thromboplastin inactivation (21–23). Essentially similar results were obtained by Thomas (24) working with brain and lung extracts as the source of thromboplastin. Thomas found that calcium ion was necessary for the thromboplastin inactivator to function most effectively, and that the thromboplastin inhibition was reversible. McClaughry (25) inactivated tissue thromboplastin with serum, and was then able by rapid sedimentation, to obtain a thromboplastic sediment and an anticoagulant supernatant, thus confirming the reversibility of the reaction. In these studies it was also determined that the known clotting factors did not cause thromboplastin inactivation. The more recent studies of Lanchantin and Ware (26) probably deal with similar or identical plasma proteins to those described by earlier investigators. These workers found that incubation of tissue thromboplastin with serum resulted in a progressive loss of thromboplastic activity after a short period of enhanced activity. If the serum had been treated with barium sulfate, this initial hyperactivity was eliminated, and the onset of inactivation was immediate. The reversibility of this reaction, and the need for calcium ions was confirmed. In all these studies on plasma protein inactivators of tissue thromboplastin, essentially similar properties have been described, save for differences in heat lability observed in the various laboratories. Nevertheless, there is no clear evidence in favor of a multiplicity of participating proteins as yet.

An antithromboplastic plasma protein of somewhat different nature has been described by Fiala (27). He noted an anticoagulant in platelet-free plasma which could be inactivated by dilution. This anticoagulant was adsorbed on a variety of surfaces, including glass, kaolin, and celite. However, it was not extractable with ether, in contrast to the agent described by Tocantins. By adsorption of plasma with barium carbonate and subsequent elution with acetic acid, an anticoagulant protein material was obtained which prolonged the clotting time of recalcified plasma, and which could be neutralized with tissue thromboplastin. It was found that this anticoagulant disappeared during the course of clotting.

Two additional types of antithromboplastic

TABLE I

Properties of reported antithromboplastins

References	Nomenclature	Dialyzable	Heat stability	Ether extractable	$BaSO_4$ absorbable	Storage stable	Protein fraction	Source
5–7, 32	Heparin and cofactor	Yes	Heparin stable cofactor inactivated at 56° C.	Heparin no; Cofactor yes	Yes	Yes	Cofactor in V of Cohn	Blood, tissues
9–19	Anticephalin; phosphatide inhibitor	Yes	Inactivated at 65° C.	Yes	Yes	No	IV_1V_{2+4} of Cohn	Blood, tissues
21–23	Antithrombo-plastin, thromboplastin inhibitor	No	Inactivated at 56–65° C.	No	No	Yes	In 50% $(NH_4)_2SO_4$ precipitate	Blood
27	Protein inhibitor		Inactivated at 65° C.	No	Yes	No		Plasma only. Lost with clotting
28–30	Sphingomyelin		Heat-stable	Yes	Yes	Yes		Tissues
31	Antithrombo-plastic protein	No	Destroyed at 65° C.	No	Yes		45–70% $(NH_4)_2SO_4$ precipitation	Muscle
Present report	Thromboplastin inactivator	No	Partially destroyed at 60°.	No	No	Yes	V of Cohn	Blood

agents have been prepared from tissues. These include the sphingomyelin group (28–30), and the antithromboplastic protein obtained from muscle by Dreyfus (31).

In Table I are summarized the properties of the various physiological agents reported to have antithromboplastic activity. The thromboplastin inactivator described in the present report probably differs from any of those previously described. With respect to its activity, it is directed against thromboplastin of blood rather than that of tissue, in contrast to the agents studied in the past. Moreover, the following characteristics serve to differentiate the present agent from those of the other authors: 1) It differs from the heparin-cofactor system in being nondialyzable, heat-stable at 56° C. and non-adsorbable on barium sulfate. 2) Unlike the anticephalin lipoid, and the sphingomyelin group of compounds, the thromboplastin inactivator is evidently a protein in the albuminoid group. 3) In contrast to the antithromboplastin of Schneider (20), Lanchantin and Ware (26) and others, the thromboplastin inactivator is active in the absence of calcium ion, and appears to have greater heat stability. 4) The thromboplastin inactivator differs from the protein described by Fiala (27) in that it is not adsorbed on the common adsorbants, it is present in both plasma and serum, and it is highly storage stable.

Because of its slow and progressive action, the thromboplastin inactivator probably functions as a removal mechanism for the blood thromboplastin rather than as an anticoagulant. In this role, its action may be comparable to the fibrinolytic system and to the adsorption of thrombin on fibrinogen. In each case the function of the reaction is the removal of a product of blood coagulation when that product no longer serves a useful physiological purpose. It would appear that the inactivators thus contrast with the anticoagulants which prevent coagulation from taking place too readily.

At present the mechanism of action of the thromboplastin inactivator is a matter of speculation. This must remain the case until there is clarification as to the nature of the thromboplastin formed by the interaction of blood elements. However, it is of interest that the thromboplastin inactivator appears to fall into the same category of proteins as do certain antithrombins (32). The possibility is suggested that thrombin may be a component of the thromboplastin complex of blood origin. Were such the case, the thromboplastin inactivation studied herein could result from thrombin inactivation.

SUMMARY

A factor has been studied which appears to inactivate the thromboplastin formed by the interaction of blood components. Measurement of this thromboplastin inactivator has been accomplished by means of the "thromboplastin inactivation test." The thromboplastin inactivator is a protein found both in plasma and in serum. It is relatively heat-stable, is not adsorbed on barium sulfate, and is found in fraction V of Cohn. The thromboplastin inactivator differs in properties and function from previously described antithromboplastins; but it has similarities to certain antithrombins. It is suggested that thrombin is a component of the thromboplastin complex, and that the present thromboplastin inactivation represents thrombin inactivation.

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A DISORDER OF BLOOD COAGULATION IN SYSTEMIC LUPUS ERYTHEMATOSUS [1, 2]

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Conley and Hartmann (1, 2) have reported the existence of a disorder of blood clotting in two patients with systemic lupus erythematosus. In both cases there were severe hemorrhagic symptoms associated with prolongation of the clotting time and the presence of a substance interfering with the interaction of thromboplastin and prothrombin.

Previous studies in this laboratory (3) had suggested that the L. E. cell factor required thromboplastin or some component thereof, in order to act on leukocytes.

A possible relationship between these two sets of data suggested itself: the L. E. cell factor, reacting in some way with thromboplastin, could inhibit thromboplastin in turn from reacting with prothrombin. If this hypothesis were valid, every blood containing the L. E. cell factor should show impaired thromboplastin function. The clotting disorder described might thus be a characteristic feature of systemic lupus.

To test this hypothesis, a systematic survey of blood coagulation in every available patient with systemic lupus erythematosus was undertaken.

MATERIALS AND METHODS

Forty-three consecutive patients with systemic lupus erythematosus were studied. Most of these were from the Wards and the Out-Patient Department of The Mount Sinai Hospital; some were private patients or patients in other hospitals.[3]

All procedures were performed on fresh blood drawn through a clean venipuncture into siliconized equipment.

The following studies were performed on each patient:

1) L. E. cell preparation: In each patient the L. E. cell was demonstrated by the clotted blood technique (4) or by incubation of serum with washed normal leukocytes (5).

2) Platelet counts were done by the method of Ottenberg and Rosenthal (6).

3) Whole blood clotting times were done by the modified Lee-White method using one tube of eleven mm. diameter with incubation at 37° C.

4) Plasma clotting times were measured by the method of Quick (7), using both platelet-rich and platelet-poor plasmas. In every case platelet-rich plasma was obtained by sedimentation of red cells without centrifugation. Platelet-poor plasma was prepared by centrifugation in silicone-coated tubes for 30 minutes at 4,500 rpm., in the Sorvall Angle Centrifuge at room temperature.

5) Prothrombin times were performed by the one-stage method of Quick (7).

6) The residual serum prothrombin activity was determined by the method of Stefanini and Crosby (8).

Where abnormalities were found, some or all of the following tests were performed:

1) The presence of an anticoagulant was shown by the ability of the blood or plasma to prolong the coagulation time of normal blood or plasma.

2) Thrombin generation tests were done by a modification of the method of Pitney and Dacie (9):

a) One ml. of platelet-rich plasma obtained without centrifugation was added to 1 ml. of 0.025 M $CaCl_2$ and 0.25 ml. of normal saline or test material in a glass tube.

b) The reaction mixture was allowed to incubate at 37° C. and at 1-minute intervals 0.1-ml. aliquots were added to 0.4 ml. fibrinogen solution.

c) The fibrinogen used was Bovine Fraction I Fibrinogen (Armour) in a concentration of 1,000 mgm. per 100 ml. of saline. Since the material contains about 50 per cent by weight of sodium citrate the actual concentration of the fibrinogen solution was in the neighborhood of 500 mgm. per 100 ml.

d) The calibration was obtained by adding 0.1-ml. aliquots of serial dilutions of topical thrombin (Parke-Davis) to 0.4-ml. portions of the fibrinogen solution. The thrombin unitage of a particular sample of plasma has been expressed as N.I.H. units per ml.

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[2] Presented (in part) at the Annual Meeting of the Eastern Section of the American Federation for Clinical Research, Boston, January, 1954.

[3] We are indebted to S. R. Karelitz, M. Levitt, S. Miller, B. S. Oppenheimer, and N. Rosenthal and to the staffs of the Brooklyn Regional Center of the Veterans' Administration, Harlem Hospital, Jewish Memorial Hospital and the New York Hospital for the opportunity to study individual patients included in this study.

TABLE I

Coagulation profile of cases showing abnormalities of clotting

Patients	Whole blood clotting time	Plasma clotting time: Platelet-rich	Plasma clotting time: Platelet-poor	Prothrombin time	Serum proth.	Platelets/mm.³
Normal values†	5–10 min.	100–180 sec.	150–250 sec.	12–14 sec.	Over 22 sec.	150,000–300,000
Severely affected						
E. L.	30	330	1,550	25.4	30	170,000
R. S.	21	*	900	20.2	9	8,000
M. M.	10	*	700	17.8	43	22,000
B. V.	15	300	720	12.8	32	80,000
B. L.	14	270	640	14.5	27	225,000
J. I.	12	195	750	16.5	38	135,000
J. H.	6	110	780	14.8	24	210,000
G. R.	8	125	400	18.2	50	255,600
M. G.	6	170	190	14.0	30	170,000
B. S.	7	105	265	20.1	69	100,000
R. V.	12	250	270	13.6	32	120,000
R. M.	10	180	225	18.9		140,000

* Severe thrombocytopenia.
† Stated normal values represent experience in this laboratory of at least 500 determinations of each type.

3) Thromboplastin dilution studies were performed by making serial dilutions of rabbit brain thromboplastin (Difco) in saline. One-tenth ml. of each dilution was added in turn to 0.1 ml. of test plasma and 0.1 ml. of 0.025 M $CaCl_2$ and clotting times measured as in the Quick prothrombin test.

4) The thrombin dilution tests were done by making serial dilutions of Topical Thrombin (Parke-Davis) from 100 to 0.5 N.I.H. units per ml. One-tenth ml. of each dilution was then added in turn to 0.1 ml. of test plasma and clotting time measured.

5) Protamine titrations were performed by making serial dilutions of protamine sulfate, adding platelet-rich oxalated plasma and 0.025 M $CaCl_2$, and measuring clotting time.

6) Fractionation of plasma was carried out by the method of Svensson (10). The identity of plasma fractions obtained was confirmed by filter paper electrophoresis.

7) Thromboplastin generation tests were carried out according to the method of Biggs and Douglas (11), modified in that barium-sulfate-adsorbed plasma was used as a source of anti-hemophilic globulin instead of alumina-treated plasma.

RESULTS

Of the forty-three patients with systemic lupus erythematosus who were studied, thirty-one showed no abnormality of the coagulation mecha-

TABLE II

Thrombin generation—Abnormal cases

(This is the table from which Figure 1 was prepared)

Time interval (*min.*)	1	2	3	4	5	6	7	8	9	10	11	12	13	14
M. M.*	0	0	0	0	0	0	0.1	0.2	0.3	0.4	2.7	0.8	0.9	0.2
J. I.*	0	0	0.2	0.3	0.4	0.5	0.9	1.9	2.2	1.2	0.9	0.5	0.2	0.2
M. G.	0	0	0	0	0.1	0.2	0.4	1.3	0.3	0.1	0	0	0	0
G. R.	0	0.4	0.4	1.3	1.9	2.0	1.7	1.6	0.9	0.8	0.4	0.2	0.2	—
E. L.*	0	0	0	0	0	0	0	0	0	0.4	0.3	—	0.3	—
R. V.	0	0	0.2	0.7	1.0	2.4	3.3	2.7	1.9	1.0	0.4	0.3	0.2	0
B. S.	0	0	0.6	1.0	1.6	0.2	1.6	0.6	0.4	0.2	0.1	0.1	0.1	0.1
R. S.*	0	0	0	0	0	0	0	0	0	0	0	0	0	0
J. H.*	0	0	0	0.4	0.9	2.4	2.5	1.2	0.7	0.4	0.1	0	0	0
B. L.*	0	0	0	0.1	0.4	0.7	1.6	3.8	2.8	2.4	1.3	0.7	0.2	0

Normal results:

1) Peak at 4 to 6 min.
2) Thrombin generated at maximum 2 to 8.7 units.
3) Measurable amounts appeared at 2 minutes in every case and fell off rapidly by 10 minutes.

* Patients in whom an anticoagulant was demonstrated (see Table III).

nism. The results obtained in the remaining twelve patients are discussed below.

1) Whole Blood Clotting Time (Table I)

In four patients the whole blood clotting time was definitely prolonged. In the remaining eight it was normal or borderline.

2) Plasma Clotting Time (Table I)

The clotting time of recalcified plasma prepared from oxalated whole blood without centrifugation was prolonged in six.

However, when the plasma was made platelet-poor the recalcification time became prolonged in three additional patients.

3) Prothrombin Times (Table I)

The prothrombin time of eight of the twelve patients was prolonged. In each case having a prolonged prothrombin time, mixture with equal parts of normal plasma failed to yield complete correction of the abnormality.

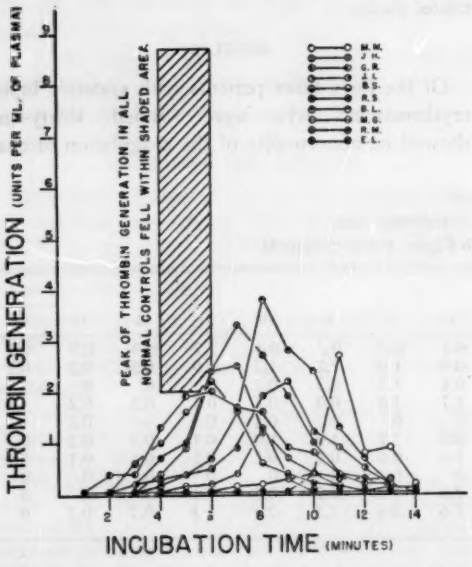
THROMBIN GENERATION (UNITS PER ML. OF PLASMA)
PEAK OF THROMBIN GENERATION IN ALL NORMAL CONTROLS FELL WITHIN SHADED AREA
INCUBATION TIME (MINUTES)

FIG. 1. DEMONSTRATING DELAY IN THROMBIN GENERATION DURING CLOTTING OF PLASMAS WHOSE SERUM PROTHROMBIN AT THE END OF ONE HOUR WAS NORMAL

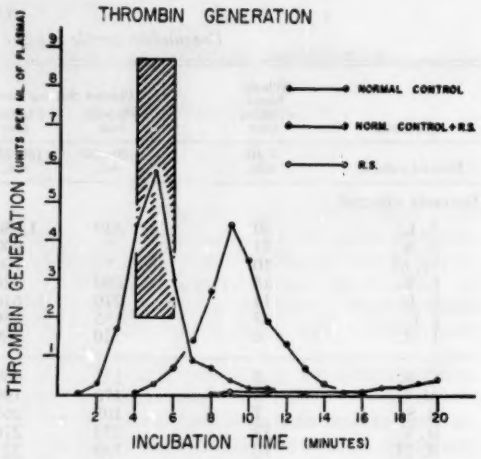

FIG. 2. THROMBIN GENERATION OF PLASMA OF PATIENT WITH SEVERE THROMBOCYTOPENIA AND ANTICOAGULANT

Shaded area represents range of peak thrombin production of 50 control plasmas. R.S. plasma alone shows almost no thrombin generation. Mixture of 80 per cent normal, 20 per cent R.S. shows delay due to anticoagulant, although total thrombin generated (*i.e.*, prothrombin consumed) is now normal.

4) Serum Prothrombin Activity (Table I)

All patients had normal consumption of prothrombin with the exception of R.S., who at the time the test was performed had a platelet count of 8,000/mm³.

5) Platelets (Table I)

The platelet count was markedly diminished in two patients, moderately so in five, and normal in five.

6) Thrombin Generation Test (Table II, Figure 1)

The thrombin generation test was performed on ten of the twelve patients, and on 50 control subjects. In three of the patients the formation of thrombin was delayed and the maximum amount generated was below normal. In five others, while there was a delay in thrombin formation, the maximum amount generated was normal. In the remaining two patients, the thrombin generation curves were low normal.

In patient R. S., who had a severe thrombocyto-

TABLE III

Recalcification times using plasma mixtures *

Normal (*ml.*)	0.2	0.15	0.10	0.05	0
Patient (*ml.*)	0	0.05	0.10	0.15	0.2
$CaCl_2$ (*ml.*)	0.2	0.2	0.2	0.2	0.2
Coag. time (*sec.*)					
E. L.	180	335	430	440	450
R. S.	90	330	490	600	900
M. M.	150	355	450	500	600
B. V.	105	225	260	255	350
B. L.	145	215	320	470	1,200
J. I.	180	285	300	500	600
J. H.	120	210	240	375	780
Control P.B. (AHG deficiency)	140	140	145	145	600

* Clotting times after recalcification of mixtures of fresh plasma from affected patients with fresh normal platelet-rich plasma. Prolongation of the clotting time of normal plasma by small proportions of the abnormal plasmas is noted.

penia, thrombin generation was both delayed and impaired. Figure 2 demonstrates the partial correction of thrombin generation by the addition of normal platelet-rich plasma. A mixture containing only 20 per cent of R. S. plasma delayed thrombin generation in the normal plasma although the peak of formation of thrombin was not materially decreased.

7) Demonstration of the Anticoagulant (Table III)

In all patients in whom a prolonged whole blood or plasma clotting time was observed, a circulating anticoagulant was demonstrated by mixtures with normal whole blood or plasma.

8) Thromboplastin Dilutions (Table IV, Figure 3)

Thromboplastin dilution studies were performed on seventy-five normal subjects and gave values shown in the shaded area of this chart. In eleven of the twelve patients under discussion there was definite prolongation of the clotting time of plasma to which diluted thromboplastin was added. Values within the normal range were obtained from one patient in this group.

9) Thrombin Dilutions

In the same seven patients in whom the anticoagulant was demonstrated, thrombin dilution studies were carried out. Abnormal findings were present in three patients. (M. M., R. S., and B. L.) When the plasma of M. M., and R. S., were stored at 4° C. overnight, their antithrombin activity had disappeared, although anticoagulant activity was unimpaired. In the case of B. L., however, both anticoagulant and anti-thrombin activity had disappeared after overnight refrigeration.

10) Protamine Titration

This procedure was carried out on three plasmas showing anticoagulant activity. No heparin-like activity could be demonstrated.

TABLE IV

Thromboplastin dilutions—Abnormal cases

(This is the table from which Figure 3 was prepared)

Coag. times	Full strength†	Dilutions of Thromboplastin (Rabbit-brain dehydrated, Difco) Times are in seconds 1:2	1:4	1:8	1:16	1:32	1:64
M. M.*	17.8	18.2	19.1	21.9	25.8	31.6	45.2
J. I.*	16.5	18.0	19.4	21.8	25.0	30.0	35.0
M. G.	14.0	15.6	17.6	21.8	26.2	33.2	54.6
G. R.	18.2	19.1	21.2	26.7	34.3	43.2	52.1
E. L.*	25.4	27.1	30.0	45.4	78.1	110.0	150.2
R. V.	13.6	14.4	17.3	21.6	30.8	37.6	
B. V.*	12.8	14.0	18.4	26.0	30.5	46.0	
B. S.	20.1	22.0	25.0	31.3	42.2	58.1	75.2
R. M.	18.9	19.3	22.3	25.6	31.5	34.2	46.4
R. S.*	20.2	23.0	30.2	39.8	50.2	63.1	78.2
J. H.*	14.8	16.4	18.8	21.4	30.6	36.0	45.8
B. L.*	14.5	16.2	20.5	27.4	33.4	41.0	50.8
Normal range	11.5–14.5	12.0–15.0	12.5–16.0	14–17.5	16–22	20–30	22–37

* Patients in whom an anticoagulant was demonstrated (see Table III).
† Equals one-stage prothrombin time.

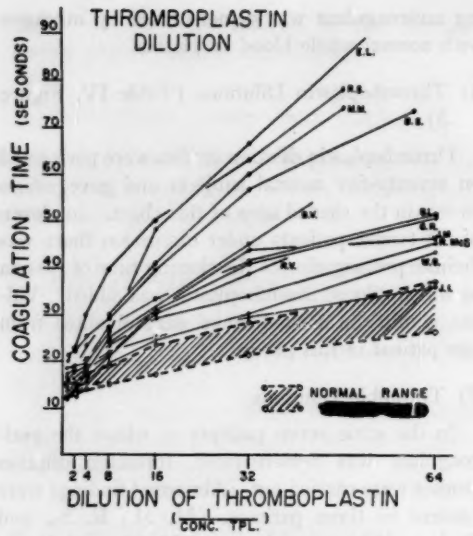

FIG. 3. DEMONSTRATING INCREASED RESISTANCE TO THE ACTION OF THROMBOPLASTIN ON THE PART OF 11 OF 12 ABNORMAL PLASMAS STUDIED

In this range of thromboplastin concentrations, plasmas with deficiencies of thromboplastic factors give normal results.

11) Thromboplastin Generation

It will be noted from Table I and Table III that in patient J. I. in whom a prolonged clotting time and anticoagulant were demonstrated the thromboplastin dilution tests were within normal limits. When a thromboplastin generation test was performed using J. I.'s plasma, it showed impaired formation of thromboplastin. When the same test was performed on plasma of E. L., and M. M., who were among the most severely affected in this group, the generation of thromboplastin was within normal limits.

12) Plasma Fractionation

Plasma from patient E. L. was found to have the highest titer of anticoagulant activity in this series (1:128).[4] Aliquots of fresh plasma from this patient were fractionated by the method of Svensson (10), after which whole plasma and each of the fractions obtained were tested for anti-coagulant activity. The fraction precipitated between 25 and 33⅓ per cent saturation of $(NH_4)_2SO_4$ was the only active one. This fraction showed mainly gamma globulin motility in paper electrophoresis.

[4] Plasma from E. L., when diluted with normal plasma to a final concentration of 1:128, showed significant prolongation of recalcification time as compared with the normal plasma alone.

DISCUSSION

From these data it would seem that seven of the forty-three patients studied showed distinct abnormality of blood coagulation. This abnormality (Table I) took roughly the same form in all seven, although it varied in degree. Marked delay of clotting of platelet-poor plasma was found in all. Most had some prolongation of whole blood coagulation time and of one-stage prothrombin time.

Although all but one (R. S.) showed apparently normal prothrombin conversion at the end of one hour, thrombin generation tests (Table II, Figure 1) showed that a delay in conversion of prothrombin was present in all of the six so studied.

Plasma mixture experiments performed on the bloods of these patients showed the presence of circulating anticoagulants in all seven. It then became important to demonstrate the sites of action of these anticoagulants. In the schema of blood coagulation, three major reactions occur: 1) The formation of thromboplastin by interaction of platelets, calcium and plasma thromboplastin precursor substances; 2) the conversion of prothrombin to thrombin by the action of thromboplastin in the presence of the two accelerator substances; and 3) the formation of fibrin from fibrinogen under the influence of thrombin. In only the third of these reactions is there any extensive knowledge of either the kinetics of the reaction or the nature of the reacting substances. Nevertheless it is possible to detect gross interference with any one of the three reactions.

Addition of varying amounts of pre-formed thrombin to a test plasma by-passes the first two reactions. Marked delay in fibrin formation (by comparison with normal controls and in the presence of normal fibrinogen concentrations) indicates the presence of a substance which inhibits the action of thrombin.

Similarly, addition of varying amounts of preformed thromboplastin to a test plasma by-passes the first reaction. A plasma which demonstrated delayed fibrin formation under the conditions of

this test may contain a substance interfering with either the second or the third reaction (if it can be shown that the defect is not due to a deficiency of any necessary substances).

By means of the thromboplastin generation test of Biggs and Douglas (11), it is possible to detect the presence of a substance interfering with the formation of thromboplastin—the first reaction of the clotting process. In this test, prothrombin-free plasma (containing some of the plasma thromboplastin precursors, but not all) is incubated with platelets and serum (containing the remaining plasma thromboplastin precursors) in the presence of calcium. At stated intervals, aliquots are removed from the reaction mixture and tested for thromboplastin potency against normal plasma. In our studies normal platelets were employed, with serum and barium-sulfate-treated plasma from the patient in question. It was possible so to manipulate the concentrations of the reagents that the critical dilution of the anticoagulant (determined previously by mixture with normal plasma) was not exceeded in the first reaction mixture, but was exceeded in the second stage of the test. In this way, although the end point of this, as of all coagulation tests, is the production of fibrin, the activity of the anticoagulant could be confined to the first coagulation reaction—the formation of thromboplastin.

The foregoing paragraphs are admittedly oversimplifications. The "thromboplastin" measured in the thromboplastin generation test of Biggs and Douglas is not the same substance as the active ingredient of dehydrated rabbit brain. And addition of thrombin to plasma does not completely by-pass the first stages of coagulation: Alexander, Goldstein, Rich, Le Bolloc'h, Diamond, and Borges (12) have shown that the anti-hemophilic factor of plasma is consumed when this reaction occurs. Nevertheless, this schema, in a crude way, allows one to characterize a circulating anticoagulant.

In six of the seven patients with circulating anticoagulants, increased resistance to the action of thromboplastin was noted (Table IV). One (J. I., Figure 3) showed insufficient evidence of this type of anti-thromboplastic activity. However, thromboplastin generation in her blood was definitely impaired, whereas thromboplastin generation of two of the most severely affected of the other patients (E. L. and M. M.) was within normal limits. On the other hand, increased anti-thrombin activity was demonstrated on only one patient (B. L.) of the seven. In this patient the anti-thrombin could have been responsible for all the abnormal findings.

In the case of patient J. I., then, the circulating anticoagulant apparently acted to prevent the formation of thromboplastin. In patients E. L., R. S., M. M., J. H., and B. V., the anticoagulants seemed to act to prevent the action of thromboplastin with prothrombin. In patient B. L., there was in addition evidence of interference with the action of thrombin.[5]

In addition to these patients, five of the remaining showed minor coagulation defects (Tables I and II, Figure 1), and an increased resistance to the action of thromboplastin. (Table IV, Figure 3). None of these five had sufficient prolongation of clotting times of blood or plasma to allow proof of the existence of circulating anticoagulants. However it seems probable that the abnormalities observed in their blood specimens were due to lesser degrees of the same disorder found in the seven cases already discussed.

Of forty-three patients studied, twelve showed disorders of coagulation other than thrombocytopenia. One of these had a circulating anticoagulant active in the first stage; eleven showed interference with the action of thromboplastin (second stage). Of these, the anticoagulant activity may have been due to an anti-thrombin in one.

Clinical significance

Only four of the forty-three patients studied had ever had hemorrhagic manifestations. All of these four had severe thrombocytopenia at one time during the course of their disease. The relationships among thrombocytopenia, anticoagulant and bleeding in these patients are summarized in Table V.

[5] In this patient repetition of studies was prevented by the ephemeral nature of the anticoagulant. Tests performed shortly after admission, when he was acutely ill gave results shown in Figures 1 and 3 and Tables I, II, III, and IV. However, retest of his blood one week later showed only slight abnormalities in coagulation, and by two weeks after initial studies, the clotting tests were all within normal limits.

TABLE V

Bleeding manifestations in systemic lupus

Patient	Duration of thrombocytopenia	Degree of bleeding	Anti-coagulant	Sites of bleeding
S. S.	Transient (splenectomy)	Severe but transient	No	Skin, Uterus
M. M.	Prolonged	Prolonged	Yes	Skin, Nose Buccal Mucosa
R. S.	Until death	Fatal	Yes	Skin, G. I. Nose ? Lungs
E. L.	Transient	Prolonged	Yes	Skin, Nose

In only one patient (E. L.) was there a hemorrhagic diathesis which could be attributed unequivocally to the presence of circulating anticoagulant. Thus the clotting disorder observed has a low order of clinical significance insofar as bleeding manifestations are concerned.

It would appear to be somewhat more important as confirmatory evidence of the diagnosis of systemic lupus erythematosus. Anomalies of clotting strictly comparable to these found in 10 of these patients have not been observed with any considerable frequency in any other disease. The literature contains reports of 33 cases of naturally occurring circulating anticoagulants. These fall into three large groups as follows:

1. Patients with congenital hemophilia who become resistant to therapy and are found to have developed an ability to neutralize anti-hemophilic globulin.

2. Young women (usually post-partum) who develop a hemorrhagic diathesis associated with prolonged clotting time and the presence of a circulating substance which can neutralize anti-hemophilic globulin.

3. A heterogeneous group of patients with acquired hemorrhagic tendencies, in whom the circulating anticoagulants appear to operate at different levels of the clotting mechanism.

Among these cases are six in which the anticoagulant was shown to act on the second stage of the coagulation process. The two cases of Conley and Hartmann (1) have already been cited. Cohen, Bonnin, and Hicks (13) have studied another such case extensively. Since completion of our studies, Frick (14) has published reports of three cases of "collagen disease" with circulating anticoagulants; two of these patients had systemic lupus erythematosus, while the third was suffering from hypersensitivity to penicillin. To these six, the present series adds five cases and another five probable (mild) cases.

All but one of these patients had systemic lupus. The apparent exception (case 2 of Frick) was a case of penicillin hypersensitivity; in this connection it should be noted that 1) a search for L. E. cells was not made, and 2) Walsh and Zimmerman (15) have reported a lupus-like picture (including L. E. cells) in three cases of penicillin hypersensitivity.

If discussion be limited to patients with clinically apparent clotting disorders, six of seven so far reported with a second-stage anticoagulant had systemic lupus erythematosus. This represents a fairly high degree of specificity (which becomes even higher if one accepts the possible identity of certain penicillin reactions with systemic lupus).

Nine of the ten patients in the present series, however, did not have clinically evident clotting abnormality; the disorder was discovered only because a systematic study was performed on every patient with a positive L. E. cell test. Similar studies have been performed, to date, on 75 control subjects, including normals, and patients with periarteritis nodosa, scleroderma, penicillin reaction, rheumatic fever, rheumatoid arthritis, carcinomatosis, leukemia, and chronic nephritis. A clotting pattern similar to that described here has been found in none of these. This does not establish the specificity of this pattern for systemic lupus erythematosus; the number of controls is too small. However, the finding of this pattern in a patient whose diagnosis is obscure must be considered as evidence for lupus.

The two remaining patients of this series showed the presence of circulating anticoagulant differing slightly from the rest. It is possible that these differences were caused by technical factors; observations on both J. I. and B. L. were unfortunately limited.

Theoretical considerations

Some evidence of the presence of an excess of anticoagulant in the blood appears, in this series, in about 28 per cent of patients with systemic lupus erythematosus, and probably, then, represents a feature of the disease. At least three other

anomalies of the plasma proteins occur with some frequency in this disease: (a) The L. E. cell factor, an abnormal constituent of the gamma globulin, present almost universally (16–18); (b) a protein capable of reacting with the phospholipids used as antigens in the standard serological tests for syphilis—this is also a constituent of the gamma globulin (19) and is present in 30 to 40 per cent of patients (20); and (c) a protein capable of reacting with red blood cells—this is also presumably a gamma globulin, since it produces a positive direct anti-globulin test, and is variously reported as being present in from 25 to 100 per cent of patients (21, 22).

The relationship of these four abnormal proteins to each other and to the disease process is, so far, obscure. A theory which has attracted many writers holds that systemic lupus is a disease of hypersensitivity; in line with this theory all the abnormal proteins found in the disease might be regarded as aberrant antibodies— respectively, to leukocytes or specific leukocyte constituents, to phospholipids, and to red blood cells. In the same way, anticoagulant substances might be antibodies to various normally present coagulation factors.

Another hypothesis of the pathogenesis of the disease stems from its practically universal association with one of these abnormal proteins—the L. E. cell factor—and from evidence that many of the anatomic lesions of the disease can be related to known effects of the L. E. cell factor (23, 24). According to this theory, presence of the L. E. cell factor would in some way be necessary for the initiation of the disease process. Other abnormal protein activities, occurring with less frequency, might then represent cross-reactions of the L. E. cell factor, detectable in some cases but not in others because of quantitative differences; or they might represent products of the disturbed metabolism.

Anticoagulant activity, directed against thromboplastin, could be an effect of the L. E. cell factor itself. This factor has been shown to require, for its action on leukocytes, the intervention of a substance derived (at least partially) from platelets, and possibly related to thromboplastin (3). If the reaction between the L. E. cell factor and leukocytes bound thromboplastin so as to prevent its reaction with other clotting factors, the L. E. cell factor would then, in effect, be an anticoagulant.

This theory provided part of the impetus for the present study. The finding that over 20 per cent of patients whose blood contains the L. E. cell factor have detectable circulating anticoagulants directed against thromboplastin activity seems to support this hypothesis. However, anticoagulants of the types found in patients J. I. and B. L. cannot be explained on this basis.

Furthermore, if the L. E. cell factor and the anticoagulant were indeed identical, the ability of a patient's serum to produce L. E. cells under optimal conditions ought to be roughly proportional to the anticoagulant activity of his plasma. It is extremely difficult to quantitate L. E. cell factor activity with any degree of accuracy or reproducibility. To the extent to which this was possible (using previously published methods [5]), the L. E. cell-producing-potential of a serum bore no relationship to the anticoagulant activity of blood from the same patient.

At the present time then, the pathogenesis of abnormal blood coagulation in systemic lupus erythematosus is unexplained.

SUMMARY

The blood coagulation mechanism has been studied in a series of 43 consecutive patients with systemic lupus erythematosus whose blood contained the L. E. cell factor.

Twelve of the 43 showed significant aberration of blood clotting. In seven of these, excess of an anticoagulant substance was demonstrated. The abnormalities in the five remaining patients were similar to, but of lesser degree than those found in the seven patients with demonstrable anticoagulant.

The pattern of abnormal coagulation in systemic lupus is as follows: 1) Slightly prolonged whole blood clotting time; 2) prolonged clotting time of platelet-poor recalcified plasma; 3) prolonged one-stage prothrombin time; 4) delayed thrombin generation but normal prothrombin consumption after one hour; 5) the presence of a substance capable of delaying the clotting time of normal blood or plasma.

In ten of the twelve patients the abnormal coagulation could be ascribed to a substance which

interfered with the action of thromboplastin, but not with the formation of thromboplastin. In one patient impaired generation of thromboplastin was found, with no interference with the action of preformed thromboplastin. In the twelfth patient, it could not be determined whether the anticoagulant activity was due to an anti-thromboplastin or an anti-thrombic substance.

The clinical significance of this clotting disorder is not great. In only 3 of the 12 affected patients was hemorrhage a prominent symptom, and in only one could the bleeding be ascribed solely to the anticoagulant activity. However, the type of clotting disorder found in 10 patients in this series seems to have a high degree of specificity for systemic lupus erythematosus.

The possible relationship of these findings to the pattern of abnormal protein metabolism in systemic lupus has been discussed.

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PURINE NUCLEOSIDE PHOSPHORYLASE ACTIVITY OF BLOOD. I. ERYTHROCYTES [1]

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The presence in animal tissues of an enzyme which splits off purines from purine nucleosides has been recognized for many years (1). This enzyme, purine nucleoside phosphorylase (PNP), has been studied by Kalckar (2–4) and has been shown to be involved in the following reaction:

ribose-1-purine + phosphate ⇌ ribose-1-phosphate + purine

Purine nucleoside phosphorylase from rat liver also possesses a certain specificity with regard to the nitrogenous bases. Inosine and guanosine are the only ribosides which undergo phosphorolysis in the presence of the enzyme. Adenosine and xanthosine are inert in the system, as are pyrimidine ribosides. Hypoxanthine and guanine are the only nitrogenous bases which are incorporated into ribosides by the enzyme. Nucleoside phosphorylases fractionated by various means catalyze the phosphorolysis of purine desoxyribosides as well as of the ribosides. Cleavage of the purine base from the pentose does not occur in the absence of phosphate but takes place readily in the presence of either phosphate or arsenate.

Purine nucleoside phosphorylase activity has been demonstrated in a number of animal tissues and in micro-organisms (4–8) but the enzyme has been studied little in the red cell. Klein (1) demonstrated its presence in the bone marrow of the calf and ox. The presence of a phosphorylase in red cells is implied in the work of Dische (9) and Tsuboi and Hudson (10). Recently, Gabrio and Huennekens (11) partially purified a nucleoside phosphorylase from red cells.

It is the purpose of this paper (a) to demonstrate the presence of PNP activity in erythrocytes; (b) to indicate the comparative activity of PNP in the red blood cells of man, dog, rabbit, pig, and chicken; and (c) to report experimentally induced changes in the PNP activity of dog erythrocytes.

METHODS

Red cell enzyme preparations were made as follows. Blood obtained by venipuncture was anticoagulated with heparin and centrifuged for 30 minutes at 3,000 r.p.m. The plasma and buffy coat were drawn off. The cells were hemolyzed with distilled water. They were not washed prior to hemolysis since this procedure did not modify the activity of the preparations.

Xanthine oxidase was prepared from raw cream [4] by the method of Ball (12) as modified by Kalckar (13). The xanthine oxidase thus prepared was standardized against a 7.6 μg. per ml. xanthine solution. In our assays, that amount of xanthine oxidase was used which in 10 minutes produced complete oxidation of xanthine to uric acid. Rat liver PNP was prepared according to the procedure of Kalckar (2).

Purine nucleoside phosphorylase activity was determined by Kalckar's method (2). In this procedure, inosine is split to hypoxanthine and ribose-1-phosphate by PNP. In the presence of an excess of xanthine oxidase, the hypoxanthine is oxidized to uric acid. The latter is then measured spectrophotometrically. In order to be certain that the end product of the reaction was uric acid and not uric acid riboside, which is known to be present in erythrocytes (14), the presence of uric acid was demonstrated by noting the changes in optical density at 292 mμ after the addition of uricase. Uricase does not act on uric acid riboside (13). All of the uric acid formed by the red cell hemolysates was present as such.

The reaction mixture consisted of 3 ml. of 0.001 M inosine in 0.06 M phosphate buffer of pH 7.4, 0.1 ml. of xanthine oxidase and 0.05 ml. of red blood cell hemolysate. These substances were added in the order described. The mixture was incubated at 36° C. to 38° C. and shaken for exactly thirty minutes. The reaction was

[1] This investigation was supported by research grants (C-2349 and C-2231) from the National Cancer Institute, National Institutes of Health, Public Health Service.

[2] Scholar in Cancer Research of the American Cancer Society (1953–1956).

[3] Student Fellow in Cancer Research (Summer, 1954).

[4] The fresh cream was generously supplied by the Cloverleaf Dairy Company, Salt Lake City, Utah.

stopped by adding 1 ml. of 20 per cent perchloric acid. After centrifugation, the absorption at 292 mμ was determined. In each case a blank was measured simultaneously, and the difference between the optical density of the reaction mixture and the blank was recorded. The blank was prepared by adding the buffer, red cell hemolysate, perchloric acid, and then the xanthine oxidase in the order named.

For convenience in reporting purine nucleoside phosphorylase activity a "unit" of activity, in terms of the assay method used, is defined as follows: one unit of PNP activity is equal to 1,000 times that amount of the enzyme which will produce one μg. of uric acid in 30 minutes at a pH of 7.4 and a temperature of 37° C. from 3 ml. of 0.001 M inosine solution in 0.06 M phosphate buffer. A standard solution of uric acid in 5 per cent perchloric acid was prepared. Under these conditions and at a wave length 292 mμ, one μg. of uric acid gave an extinction of 0.056. Thus,

$$\frac{\text{PNP units}}{\text{ml. packed RBC}} = \frac{\text{optical density}}{0.056} \times \frac{4.15\ (\text{final vol.})}{1{,}000} \times \frac{\text{dilution of hemolysate}}{\text{vol. of hemolysate (.05 ml.)}}$$

or,

$$\text{PNP units/ml. packed RBC} = 1.48 \times \text{optical density} \times \text{dilution of hemolysate.}$$

The volume of packed red blood cells and reticulocytes were determined by methods described previously (15).

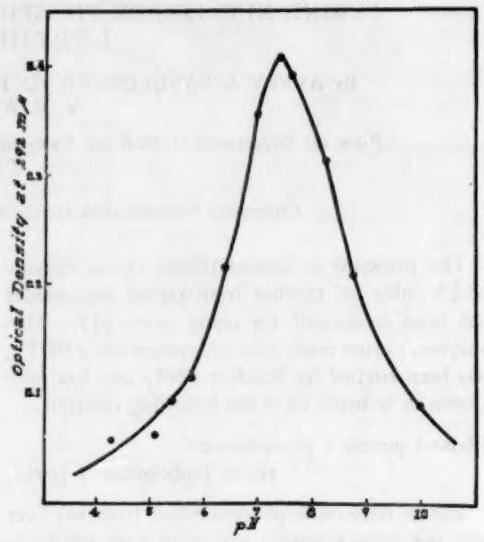

FIG. 1. THE EFFECT OF pH ON PNP ACTIVITY OF HUMAN RED BLOOD CELLS

Phosphate buffer and 0.05 ml. of a 1:100 hemolysate of red cells were used.

RESULTS

Characteristics of erythrocyte purine nucleoside phosphorylase

In the absence of red blood cell hemolysate xanthine oxidase did not split inosine. When xanthine oxidase was incubated with the hemolysate of either human or dog erythrocytes, there were only negligible changes in the optical density at 292 mμ. In none of the preparations used could any uricase activity be demonstrated by incubating the erythrocyte hemolysate with uric acid in glycine buffer of pH 9.4. The determination of uricase activity was necessary since the presence of uricase would nullify the results of these experiments. No xanthine oxidase activity could be demonstrated in the red cells of man or dog when the hemolysates were incubated with a solution of xanthine.

When glycylglycine buffer, pH 7.2 and 0.05 M, was used in the reaction, replacing the phosphate buffer, no splitting of inosine could be demonstrated. The addition of phosphate buffer resulted in an abrupt breakdown of inosine by the PNP of the red cell hemolysate. When arsenate buffer was used in the reaction in place of phosphate buffer, PNP activity was demonstrated (Table I).

TABLE I

PNP activity in phosphate buffer and in arsenate buffer *

Time min.	Phosphate buffer	Arsenate buffer
15	14	8
30	30	19
60	59	36
120	120	74
180	179	112

* The reaction mixture consisted of 3 ml. of 0.001 M inosine in 0.06 M phosphate buffer (pH 7.4) or in 0.05 M arsenate buffer (pH 7.4), 0.1 ml. of xanthine oxidase and 0.05 ml. of a 1:200 dilution of red cell hemolysate. The results are expressed in "units" of PNP activity.

The effect of varying pH on the PNP activity of red cells is shown in Figure 1. The optimum pH for this reaction is 7.4.

The effects of time and of different concentrations of inosine, phosphate, and xanthine oxidase on PNP activity of red cells are shown in Figure 2. Inosine was converted to uric acid in a linear fashion over a three-hour period. An inosine concentration of 0.001 M was used in these experiments and the activity at this concentration falls on the plateau part of the PNP activity-sub-

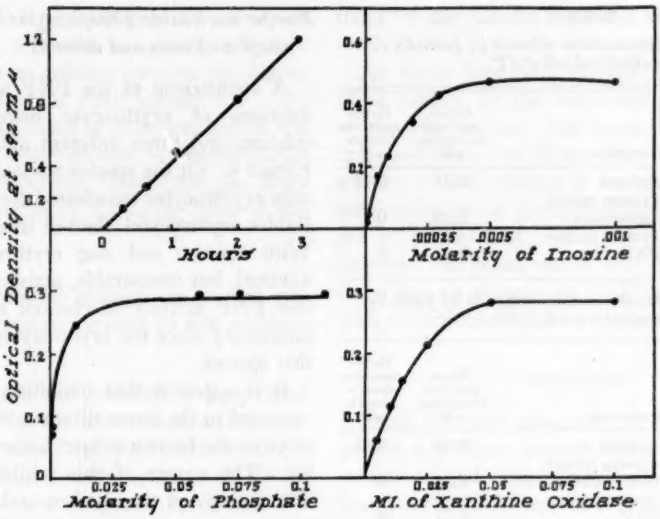

FIG. 2. THE EFFECTS OF TIME AND THE INFLUENCE OF DIFFERENT CONCENTRATIONS OF INOSINE, PHOSPHATE OR XANTHINE OXIDASE ON HUMAN RED CELL PNP ACTIVITY

Five hundredths ml. of a 1:100 hemolysate of red cells was used in all experiments.

strate molarity curve shown in Figure 2. A phosphate concentration of 0.06 M to 0.12 M was optimal. It is apparent from Figure 2 that, for every new preparation of xanthine oxidase, the optimal amount to be used should be determined.

Temperatures below 36° C. resulted in decreased PNP activity. For example, at 26° C., about one-fifth of the activity was present compared to that at 37° C.

When the rate of degradation of inosine by red blood cell PNP was compared with that of hypoxanthine desoxyriboside under identical conditions, it was found that inosine is split more readily than the desoxyriboside (Figure 3).

No phosphorolysis or hydrolysis of xanthosine or the ribosides of pyrimidines (uridine, cytidine) could be demonstrated with the hemolysates of red cells. The methods of Lampen and Wang (16) were used to follow the reactions with uridine and cytidine.

When hemolysates of red cells were incubated in 0.05 M glycylglycine buffer at pH 7.2 with adenosine, adenosine deaminase activity could be demonstrated. When the red cell hemolysate was incubated with xanthine oxidase, phosphate buffer, and adenosine as a substrate, some splitting of adenosine occurred. In a single experiment, the activity with adenosine as a substrate was about one-fifth to one-tenth of that which occurred when inosine was used as the substrate.

Partially purified PNP was prepared by lysing 10 ml. of packed red cells with 200 ml. of water. Solid ammonium sulfate was added to the hemolysate. The final concentration of ammonium sulfate was 1.6 M. The precipitate was discarded

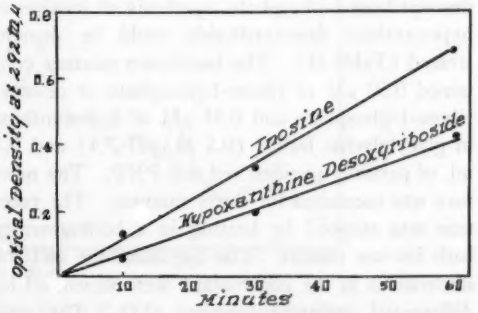

FIG. 3. THE CLEAVAGE OF INOSINE AND HYPOXANTHINE DESOXYRIBOSIDE BY RED CELL HEMOLYSATE

TABLE II

Synthesis of hypoxanthine riboside by partially purified red cell PNP

Experiment no.	Description	Hypoxanthine remaining μM	Hypoxanthine riboside μM
1	Complete system	0.23	0.12
2	Complete system minus ribose-1-phosphate	0.35	0
3	Complete system minus red cell PNP	0.35	0

Synthesis of hypoxanthine desoxyriboside by partially purified red cell PNP

Experiment no.	Description	Hypoxanthine remaining μM	Hypoxanthine desoxyriboside μM
4	Complete system	0.30	0.05
5	Complete system minus desoxyribose-1-phosphate	0.35	0
6	Complete system minus red cell PNP	0.35	0

and solid ammonium sulfate was added to the supernatant solution until the final concentration of ammonium sulfate was 2.4 M. The solution was then centrifuged and the non-dialyzed precipitate was used for assay. All fractionation steps were carried out at room temperature. The final precipitate was dissolved in water and diluted to the volume of the original hemolysate. The activity of the original hemolysate was then compared to the activity of the partially purified preparation. One ml. of this solution contained approximately 25 times the activity of one ml. of the original hemolysate.

When the partially purified PNP of red corpuscles was incubated with ribose-1-phosphate or desoxyribose-1-phosphate, synthesis of inosine or hypoxanthine desoxyriboside could be demonstrated (Table II). The incubation mixture contained 0.50 μM of ribose-1-phosphate or desoxyribose-1-phosphate and 0.35 μM of hypoxanthine in glycylglycine buffer (0.5 M, pH 7.4) and 0.1 ml. of partially purified red cell PNP. The mixture was incubated for thirty minutes. The reaction was stopped by heating in a boiling water bath for one minute. The hypoxanthine and the nucleosides in the supernatant were measured by differential spectrophotometry (13). The synthesis of inosine was of greater magnitude than that of its desoxyribose counterpart.

Purine nucleoside phosphorylase activity of erythrocytes of man and animals

A comparison of the PNP activity at various dilutions of erythrocyte hemolysate in man, chicken, and three different animals is shown in Figure 4. Of the species studied, porcine and human erythrocytes manifested the greatest activity. Rabbit erythrocytes showed intermediate activity. With chicken and dog erythrocytes there was minimal, but measurable, activity. The relatively low PNP activity of chicken red blood cells is interesting since the erythrocytes are nucleated in this species.

It is apparent that inhibition of PNP activity occurred in the lower dilutions of the cell hemolysates of the human subjects, the pig, and the rabbit. The nature of this inhibition is unknown. The activity of the chicken and dog erythrocytes was too low to permit any conclusions concerning inhibition.

The purine nucleoside phosphorylase activity of a 1:200 dilution of the erythrocyte hemolysate for six normal human subjects was found to range between 87 and 112 units per ml. of packed RBC with a mean value of 99 units. A value of 81 units per ml. packed red cells was observed in a patient with acquired hemolytic anemia and a reticulocyte count of 11.5 per cent. A 1:200 dilution of an erythrocyte hemolysate from a patient with paroxysmal nocturnal hemoglobinuria and a retic-

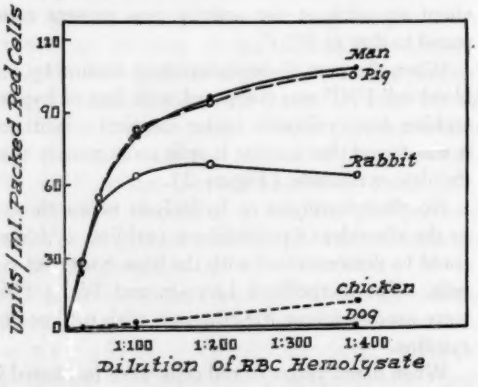

FIG. 4. VARIATION OF RED CELL PNP ACTIVITY OF VARIOUS SPECIES AT DIFFERENT DILUTIONS OF HEMOLYSATES

The data presented represent the averages of six humans (four male, two female), one pig, two rabbits, one chicken, and seven dogs.

ulocyte count of 23.1 per cent gave an activity of 115 units. Thus, it would seem that in human subjects there was no relation between the degree of reticulocytosis and the PNP activity.

No PNP activity could be demonstrated in the stroma of the red cells obtained by centrifugation at 8,000 r.p.m. (American Instrument Co. High Speed Angle Centrifuge) following lysis of the cells with water.

Experimentally induced changes in purine nucleoside phosphorylase activity of dog erythrocytes

When acute anemia was produced in dogs, either by phlebotomy or by the intravenous administration of phenylhydrazine, the PNP activity of the erythrocytes increased simultaneously with the increase in reticulocytes (Figures 5, 6, and 7). However, the PNP activity decreased to the preanemic levels more slowly than did the reticulocytes.

The PNP activity of dog plasma also increased considerably during the hemolytic phase following phenylhydrazine administration (Figure 5). In those animals which survived, the PNP activity of the plasma returned slowly to normal levels.

An acute hemolytic anemia was produced in three rabbits by the intravenous administration of phenylhydrazine. The mean reticulocyte levels increased from 3 to 37 per cent. The mean PNP activity (1:200 dilution of hemolysate) before the administration of phenylhydrazine was 51 units per ml. packed RBC and at the time of the reticulocytosis mentioned above was 52 units. Thus, there was no increase in PNP activity associated with the reticulocytosis in this species.

DISCUSSION

From the data presented, it appears that the purine nucleoside phosphorylase of red cells is similar to that which has been described in the tissues of animals and in micro-organisms (2–8, 17, 18). Whether or not it is identical with tissue PNP cannot be stated from the information available. Although the enzymatic action of PNP seems to be clear, the role of the enzyme in the metabolism of the erythrocyte is unknown.

The species differences in PNP activity of red cells are difficult to explain. They may be re-

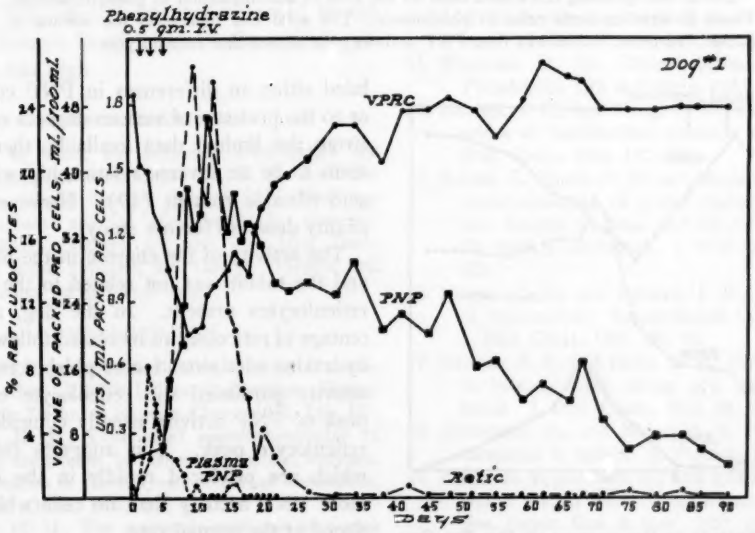

FIG. 5. CHANGES IN PNP ACTIVITY OF DOG RED CELLS DURING AND AFTER THE PRODUCTION OF ACUTE HEMOLYTIC ANEMIA BY THE ADMINISTRATION OF PHENYLHYDRAZINE

Note the high PNP activity in the plasma during the height of hemolysis.

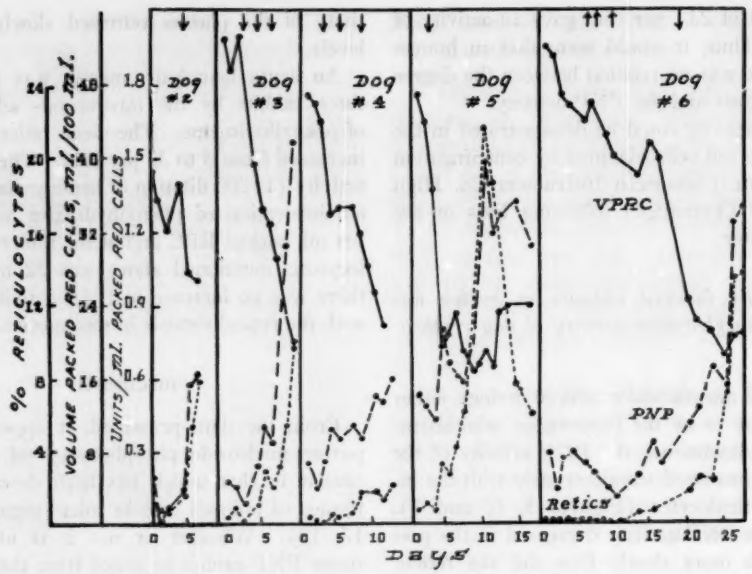

FIG. 6. CHANGES IN PNP ACTIVITY OF DOG RED CELLS DURING ACUTE ANEMIA

Note that there was only slight reticulocytosis and moderate elevation of PNP activity in Dog No. 4. The high initial activity in Dog No. 5 may have been due to the fact that the animal had been made acutely anemic ten weeks prior to the experiment. Dog No. 6 showed slight reticulocytosis after bleeding. The PNP activity rose only to a slight extent. When marked anemia and reticulocytosis were produced with phenylhydrazine, the PNP activity increased markedly.

The arrows pointing downward refer to the time of administration of phenylhydrazine. Those directed upwards refer to phlebotomy. The solid line represents the volume of packed red cells; the broken line, PNP activity; the dotted line reticulocytes.

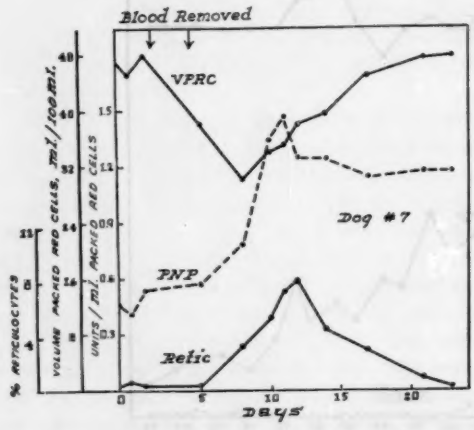

FIG. 7. THE EFFECTS OF PHLEBOTOMY ON DOG RED CELL PNP ACTIVITY

Note the persistence of high activity even after the percentage of reticulocytes returned to a normal level.

lated either to differences in PNP concentration or to the presence of various degrees of inhibition. From the limited data available, there does not seem to be an inverse relationship with the uric acid riboside content (19). However, this possibility deserves further study.

The activity of the enzyme in the blood of man and the rabbit was not related to the per cent of reticulocytes present. In the dog, as the percentage of reticulocytes increased following phenylhydrazine administration or phlebotomy, the PNP activity paralleled the reticulocyte count. The peak of PNP activity usually coincided with the reticulocyte peak. This suggests that red cells which are produced rapidly in the dog possess more PNP activity than do cells which are produced at the normal rate.

It has been demonstrated in the rabbit and the rat that cells which are rapidly produced are destroyed in a random fashion and survive in the

circulation for a shorter time than do cells which are produced at the normal rate (20, 21). If the same holds true for the dog, whose normal erythrocyte life span is about 100 days (22), the slow exponential type of decay curve of PNP activity over a period of about 70 days depicted in Figure 5 suggests that the increased PNP activity remained in the rapidly produced cells of the dog for the duration of their survival.

SUMMARY

1. Purine nucleoside phosphorylase activity, similar to that of other animal tissues, has been demonstrated in the erythrocytes of man, dog, rabbit, pig, and chicken.

2. The purine nucleoside phosphorylase activity was greatest in the erythrocytes of man and the pig. Dog and chicken erythrocytes showed much less activity. The activity of rabbit erythrocytes was intermediate between that of these two groups.

3. The purine nucleoside phosphorylase activity of dog erythrocytes was enhanced markedly following the production of acute anemia by the administration of phenylhydrazine or phlebotomy. Under similar circumstances no increase in PNP activity was noted in rabbit red cells. An increase in PNP activity was not observed in red cells from human blood in which the reticulocyte percentage was high.

ACKNOWLEDGMENTS

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THE ENHANCEMENT OF PERIPHERAL GLUCOSE UTILIZATION BY GLUCAGON

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During the past 30 years evidence has been accumulating which suggests that glucagon (the hyperglycemic-glycogenolytic factor of the pancreas) is a true hormone and that it is produced in the Islets of Langerhans (1, 2). Its purification and crystallization were finally accomplished in 1953 by Staub, Sinn, and Behrens (3). The crystalline material is a simple protein with a molecular weight of approximately 4,200 and an amino acid composition which differs in important ways from that of insulin (4). This establishes glucagon as an entity distinct from insulin.

Crystalline glucagon is an extremely active substance. In the cat, 0.05 γ per Kg. body weight causes a significant rise in blood sugar which lasts 25 minutes (4). Sutherland, Cori, Wosilait, and Rall (5, 6) have shown that glucagon stimulates the synthesis of active phosphorylase in the liver. This enzyme catalyzes the rate-limiting reaction (glycogen $\rightleftarrows$ glucose-1-phosphate) in the transformation of glycogen to glucose. The effect of glucagon on liver phosphorylase adequately explains its well-known hyperglycemic-glycogenolytic action.

With respect to its role in carbohydrate metabolism, some investigators look upon glucagon as an insulin antagonist (1, 2, 7) while others consider it an insulin synergist (8, 9). Still others are skeptical of its hormonal nature (10, 11). In the present paper experiments are reported which indicate that glucagon, in addition to mobilizing liver glycogen, increases the peripheral utilization of glucose.[1]

METHODS

Human experiments. Thirty-two male subjects (ages 21 to 47) considered normal with respect to carbohydrate metabolism were studied. All were hospital patients on high caloric and carbohydrate intakes for at least three days before being studied. The test procedure was begun in the morning after a 14-hour fast. Soon after awakening the subjects were placed at bed rest for 30 to 60 minutes in the testing room before the experiment was begun. A 10 per cent solution of glucose was infused intravenously at a constant rate (250 to 370 mg. per min. in different subjects) by means of a constant infusion pump (Bowman) for periods of 120 to 145 minutes. In 12 subjects glucagon[2] (0.7 to 1.0 mg.) was added to the perfusate following an initial period of 40 to 60 minutes with glucose alone. In 12 subjects the glucose infusion rate was doubled after 50 minutes and after 90 minutes the initial rate was resumed in order to simulate the hyperglycemia caused by glucagon. Two subjects received glucose at a constant rate for 2 hours. The initial control data on 8 other subjects who subsequently received other hormones were also used in the analysis of data.

Glucose determinations (Nelson-Somogyi Method) (12) were done on venous (indwelling needle in antecubital vein) and capillary (finger) blood from the same arm at 5 or 10-minute intervals, using 0.2-ml. samples (30 to 40 samples from each subject). Venous samples were drawn without stasis and analyzed in duplicate. (The average difference between duplicate determinations was 2 per cent.) The antecubital vein carries venous drainage from the deep and superficial tissues of the forearm (13–15), while capillary blood has the same glucose content as arterial blood (16–18).

Sodium space was determined in 8 subjects receiving glucagon and 12 subjects receiving glucose alone using radiosodium (Na^{22}) which was added to the infusion fluid. Plasma samples (drawn along with samples for glucose determination) were assayed for radioactivity in a R-C Scientific scintillation well type counter. The method used to calculate sodium space by this technique will be described elsewhere (19).

Dog experiments. The effect of glucagon on arteriovenous glucose differences in the dog was also studied. Six experiments were done in five normal dogs, and nine experiments in three depancreatized dogs. The normal animals were studied under Pentobarbital Sodium anes-

[1] Glucose utilization is used to mean the disappearance of blood glucose.

[2] Preparations No. 208-158B-214 and No. 208-158B-214A kindly supplied by Drs. O. K. Behrens and W. R. Kirtley of the Eli Lilly Company. These preparations have a glucagon potency of 50 per cent of the crystalline standard and an insulin content of between 0.05 to 0.005 unit per mg.

thesia, after a fast of 16 hours. The depancreatized dogs were tested (under anesthesia) 4 to 16 hours after their last dose of regular insulin and food. Glucagon in doses of 0.072 to 1.5 mg. was administered intravenously, either as a single injection or by continuous infusion. Blood samples were drawn without stasis from indwelling needles in the femoral artery and vein. Glucose determinations were done on 1-ml. (normal dogs) or 0.5-ml. (depancreatized dogs) samples in duplicate.

RESULTS

Human experiments

Tables I, II, and III contain the arteriovenous glucose data from the 32 subjects. Two types of response to glucagon were observed. In 11 subjects, blood sugar fell after reaching a peak level, despite the constant infusion of glucagon (Figure 1-A). In one subject the peak glucose levels persisted throughout the test period. In all subjects glucagon, in addition to producing a sharp rise in blood sugar levels resulted in a marked increase in arteriovenous (A-V) glucose differences. In the 12 subjects in whom the glucose infusion was doubled following the control period, the arterial glucose concentrations closely simulated those observed in the subjects receiving glucagon. However, the A-V glucose differences were much less (Figure 1-B). The A-V/A [3] values of the 12 glucagon periods were compared statistically with those of the 32 control glucose

[3] A-V/A = arteriovenous glucose difference/arterial glucose concentration. This serves as an index of the peripheral utilization constant of glucose (see appendix for derivation).

TABLE I

Glucagon cases *

Patient	Age		Time in minutes 0	10	20	30	40	50	60	70	80	90	100	110	120	130	140
B. D.	30	A†	86	98	107	112	115	117	118 ↑	137	172	187	178	162			
		V†	79	88	95	100	102	105	106 |	107	144	150	146	133			
W. D.	26	A	85	104	118	129	137	142 ↑	147	173	197	200	195	188			
		V	85	101	111	118	124	127 |	129	152	167	174	164	130			
K. R.	21	A	78	101	111	118	124	129 ↑	142	167	183	183	177	169	165		
		V	77	93	103	110	116	121 |	125	146	158	160	157	149	137		
C. C.	46	A	90	97	102	107	110 ↑	114	136	151	160	167	171	175	177	176	
		V	89	95	100	105	108 |	112	125	139	147	155	159	164	167	165	
R. L.	24	A	80	89	95	99 ↑	101	150	184	207	222	230	211	193	177	164	
		V	77	86	92	96 |	98	135	170	195	207	212	192	175	158	134	
J. D.	36	A	85	96	103	107	109 ↑	126	153	169	176	172	156	144	133	122	112
		V	82	94	100	102	103 |	111	139	157	158	150	142	133	122	113	106
R. B.	47	A	87	98	106	113	118 ↑	127	147	164	172	165	161	157	153	148	143
		V	82	92	98	94	106 |	108	125	139	141	135	120	118	116	114	114
R. R.	29	A	99	109	116	120	125 ↑	141	167	190	198	203	207	219	187	178	173
		V	97	106	113	117	122 |	133	154	174	189	194	192	188	149	143	139
F. R.	34	A	80	93	99	101	102 ↑	119	155	179	194	189					
		V	80	91	95	96	96 |	105	137	161	177	174					
G. N.	35	A	110	130	142	149	155 ↑	158	164	209	231	243	228	213	202	198	
		V	105	123	136	144	150 |	153	157	194	214	215	196	189	183	182	
R. T.	30	A	91	105	113	119	122 ↑	138	168	186	191	178	161	145	131	125	122
		V	87	102	108	112	114 |	124	148	159	163	149	136	123	112	104	99
G. W.	32	A	78	89	99	108	114 ↑	131	166	198	214	217	206	184	170	161	156
		V	76	87	97	106	112 |	117	147	167	175	178	172	157	144	135	131

* Blood sugar values at 10-minute intervals taken from curves constructed from determinations on samples (30 to 40 per subject) drawn at staggered time intervals which varied in the different subjects.

† A—Capillary blood sugar (mg. per cent), V—Antecubital vein sugar (mg. per cent).

↑—Beginning of glucagon infusion.

TABLE II

Glucose hyperglycemia cases *

Patient	Age		Time in minutes 0	10	20	30	40	50	↑ 60	70	80	90	↓ 100	110	120	130
G. N.	35	A†	96	112	123	131	137	142	166	183	197	208	199	193	190	187
		V†	92	104	114	122	128	133	151	168	182	194	196	190	187	185
H. C.	33	A	82	94	101	106	109	132	144	152	157	144	137	131		
		V	79	89	96	101	104	127	138	144	148	141	134	128		
R. W.	27	A	84	96	104	109	115	126	142	151	157	160	136	121	111	
		V	79	92	101	107	113	114	128	137	144	148	124	103	95	
F. R.	34	A	87	100	109	116	121	125	144	157	168	177	167	159	153	148
		V	85	96	105	111	116	121	133	144	155	165	159	153	148	143
H. R.	42	A	92	105	114	121	126	130	151	165	174	181	173	166	160	156
		V	88	101	112	119	125	129	144	159	170	179	170	162	156	153
A. S.	22	A	86	100	108	114	118	122	142	156	167	176	165	154	144	134
		V	85	98	107	113	117	121	138	152	163	166	155	145	136	129
B. M.	47	A	93	106	115	121	126	130	156	172	183	193	181	171	162	
		V	89	103	111	117	121	124	140	154	162	170	162	151	141	
T. B.	35	A	84	100	109	113	115	116	135	152	165	173	157	143	133	127
		V	81	95	104	107	107	108	122	137	149	160	143	127	114	108
C. H.	35	A	81	108	121	129	135	139	169	186	200	212	202	194	187	181
		V	81	104	116	125	131	135	164	183	197	209	200	192	186	180
V. F.	37	A	81	95	105	112	117	121	145	159	168	174	164	155	146	138
		V	78	90	98	102	106	108	118	140	153	161	150	141	135	133
E. A.	27	A	84	98	109	116	121	124	143	154	164	172	150	135	127	122
		V	83	92	99	104	108	110	128	139	147	154	133	119	112	109
V. B.	31	A	89	112	123	130	135	139	165	180	192	202	184	171	164	160
		V	87	107	118	126	131	134	157	171	183	193	178	167	161	157

* Blood sugar values at 10-minute intervals taken from curves constructed from determinations on samples (30 to 40 per subject) drawn at staggered time intervals which varied in the different subjects

† A—Capillary blood sugar (mg. per cent), V—Antecubital vein sugar (mg. per cent).

↑—Beginning of increased rate of glucose infusion. ↓—Resumption of initial speed of glucose infusion.

TABLE III

Miscellaneous control cases *

Patient	Age		Time in minutes 0	10	20	30	40	50	60	70	80	90	100	110	120
C. F.	45	A†	102	121	135	145	154	161	167	171	174	176	178	179	180
		V†	100	118	132	142	151	158	164	168	170	172	173	174	175
P. H.	30	A	95	112	124	133	140	146	150	154	156	159	162	164	
		V	88	104	115	125	132	139	143	147	149	151	154	156	
G. M.	22	A	86	110	128	141	150	157	161						
		V	83	107	124	136	145	152	156						
H. C.	33	A	86	103	114	121	126	130	133						
		V	83	98	109	116	121	124	127						
D. Z.	24	A	87	104	116	125	131	135	137						
		V	85	100	110	117	123	127	131						
R. G.	36	A	87	109	121	127	131	133							
		V	85	104	115	121	125	128							
R. B.	29	A	97	109	120	129	134	138							
		V	92	104	114	122	127	131							
C. J.	26	A	87	102	116	126	134	139							
		V	82	97	109	117	123	127							

* These subjects received glucose infusion alone at one constant rate. Data treated as in Tables I and II.

† A—Capillary blood sugar (mg. per cent), V—Antecubital vein sugar (mg. per cent).

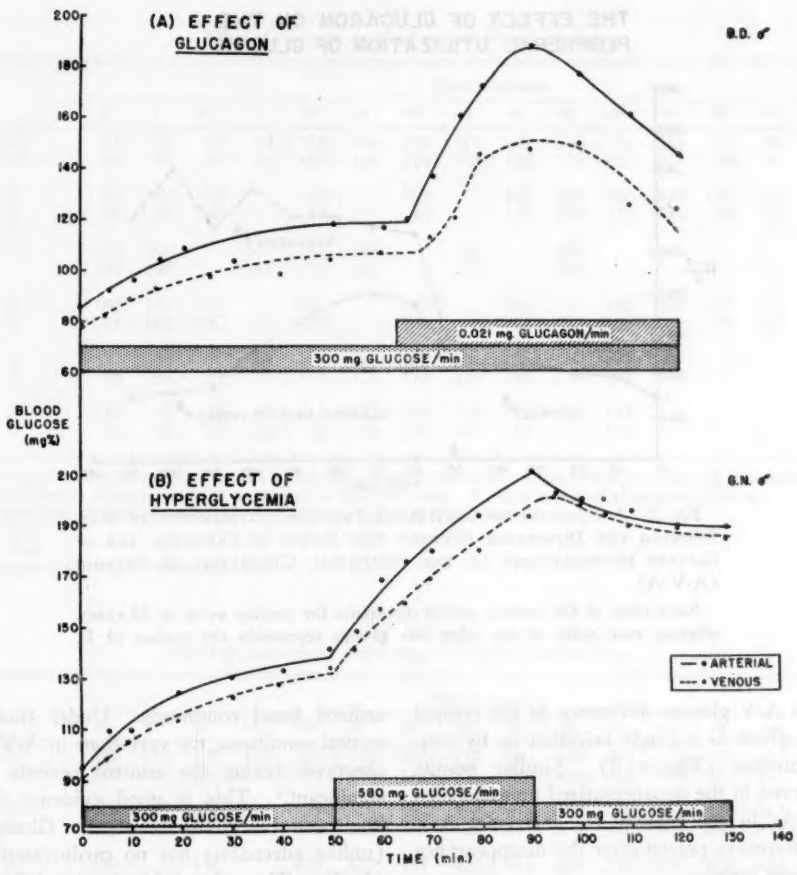

FIG. 1. COMPARISON BETWEEN THE EFFECTS OF (A) GLUCAGON AND (B) HYPERGLYCEMIA DUE TO GLUCOSE ON ARTERIAL AND VENOUS GLUCOSE CONCENTRATIONS IN MAN

periods. The data were subjected to a median test employing a 2×2 contingency table (20). The resulting chi^2 values showed that the increase in A-V/A values during the glucagon periods was highly significant ($P = .001$). A similar statistical analysis was applied to the differences between the control and experimental periods for both the glucagon and glucose hyperglycemia cases. This revealed that glucagon caused a significantly greater ($P = .001$) increase in A-V/A than did glucose hyperglycemia. A comparison between the three groups of experimental periods is shown in Figure 2. These experiments demonstrate that the increased A-V glucose differences observed with glucagon cannot be attributed to the increase in blood glucose levels *per se*. Further, it should be noted that the increased A-V/A values caused by glucagon persist throughout the test period despite the fall of blood sugar toward pre-glucagon levels in 11 of the 12 subjects.

Sodium space did not change following glucagon administration or when the rate of the glucose infusion was doubled. The mean value for the 20 subjects tested was 21.35 ± 3.35 per cent of body weight.

Dog experiments

Tables IV and V contain the data from the animal experiments. Glucagon causes a marked

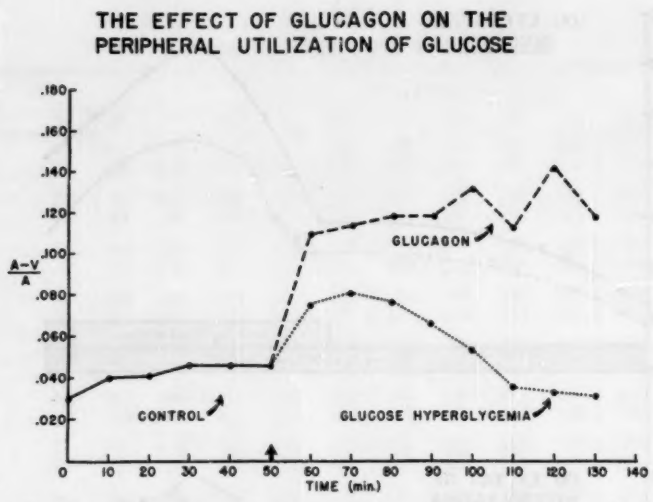

FIG. 2. A SUMMARY OF CONTINUOUS INFUSION EXPERIMENTS IN MAN SHOWING THE DIFFERENCE BETWEEN THE EFFECT OF GLUCAGON AND OF GLUCOSE HYPERGLYCEMIA ON THE PERIPHERAL UTILIZATION OF GLUCOSE (A-V/A)

Each point of the control period represents the median value of 32 cases, whereas each point of the other two groups represents the median of 12 cases.

increase in A-V glucose difference in the normal dog when given as a single injection or by continuous infusion (Figure 3). Similar results were observed in the depancreatized animal (Figure 4). As in the human, the increased A-V glucose differences persist after the disappearance of the hyperglycemia.

DISCUSSION

Previous work from this laboratory, using a single glucose injection technique, suggested that glucagon causes a marked increase in A–V glucose differences in man (21). Recently, Van Itallie, Morgan, and Dotti (22) have reported experiments which support this finding. Both of these studies depended on the assumption that blood flow and A-V glucose differences remain constant from day to day in a given individual. Actually, blood flow and A-V glucose differences may vary considerably in a given individual from day to day (23, 24). The present studies were designed to minimize these variables. Thus, both the control and hormone test periods were run in sequence within a 2½-hour period under standardized basal conditions. Under these experimental conditions, the variations in A-V/A values observed during the control periods were not significant.[4] This is good evidence that blood flow was essentially constant. Glucagon itself (unlike adrenalin) has no cardiovascular effects (1, 2). Thus the A-V glucose differences observed in the present experiments may be considered proportional to glucose utilization in the forearm and hand.

The relationship of glucose utilization to the height of the arterial blood sugar is pertinent to this study, since glucagon causes a marked rise in arterial glucose concentration. The glucose infusion experiments indicate that peripheral utilization of glucose is proportional to arterial glucose levels in the range between 80 and 210 mg. per cent in agreement with previous work (16–18). Glucagon results in significantly greater glucose utilization at comparably elevated glucose levels.

[4] A Rank Sign Test (25) of the significance of the variation in successive 10-minute time periods revealed no significant variation beyond the first 10-min. interval. This appears obvious from Figure 2.

TABLE IV

*Effect of glucagon on blood sugar * of normal dogs*

Dog number		Time in minutes															
		0	5	10	15	20	25	30	35	40	45	50	60	70	80	90	100
S† 564	A‖	81	82	82	84 ↑	116	146	160	167	159	146	134	112	97	92		
	V‖	80	80	81	82 |	107	125	140	148	146	133	122	101	88	83		
C 580	A	87	88	89		89 ↑		102		142	148	143	129	115	105	96	
	V	85	89	87		88 |		95		120	138	132	116	99	82	79	
S 435b	A	115	108		109 ↑	146	171		195		188						
	V	103	103		103 |	129	151		172		162						
C 563	A	102	102	101		101		102 ↑		146		190	202	187	171	157	144
	V	101	100	100		100		100 |		125		165	164	156	148	138	128
S 529‡	A	92	86	85	88 ↑	124	145	157	165	163	156	149					
	V	85	83	85	86 |	102	124	132	139	139	137	134					
S 529§	A	88	91	88	89 ↑	115	125	128	126	123	120	115					
	V	82	86	83	86 |	93	100	104	107	110	111	110					

* Blood sugar values taken from curves which were constructed from values taken at staggered time intervals which varied in the different animals, and are expressed in mg. per cent. ↑—Beginning of glucagon infusion.
† S—Single injection of glucagon, C—Continuous infusion of glucagon.
‡ 48-hr. fast.
§ 86-hr. fast.
‖ A—Femoral artery, V—Femoral vein.

TABLE V

*Effect of glucagon on blood sugar * of depancreatized dogs*

Dog number		Time in minutes															
		0	5	10	15	20	25	30	35	40	45	50	60	70	80	90	100
S† 560‡	A¶	362	352	351	357	368 ↑	410	441	454	463	470	473	473	453	433	417	404
	V¶	356	356	351	353	357 |	386	413	430	440	447	453	454	432	411	396	384
S 435a‡	A	270	272	274	275	277 ↑	316	353	379	394	404	410	418	419	413		
	V	272	279	280	281	282 |	288	305	322	337	350	362	381	393	399		
C 435a‡	A	439		431		428	427	427	425 ↑	473	499	519	565	600	584	560	544
	V	423		416		410	412	415	423 |	431	450	473	506	526	529	525	518
S 560‡	A	310		316		322		328 ↑	345	374	390	400	414	420	309	412	400
	V	302		306		309		311 |	314	338	355	365	383	390	393	392	388
C 543‡	A	235	230	233	234	230 ↑		286	310	323	332	337	334	329	323	316	309
	V	232	233	234	234	230 |		252	283	304	315	324	324	322	317	310	303
C 560§	A	75		79		82	85 ↑	128	175	199	215	226	239	248	253	257	260
	V	72		74		76	82 |	96	129	157	174	185	203	215	225	231	234
S 435a‖	A	62	60	56	58	60 ↑	128	200	255	280	300	317	338				
	V	60	58	53	51	48 |	95	147	190	217	229	241	257				
S 435a‖	A	87	90	89	89 ↑	119	139	152	162	168	173	178	184				
	V	80	72	78	84 |	99	112	124	131	142	148	152	157				
S 435a‖	A	69	78	89	73 ↑	114	143	158	167	177	184	189	194				
	V	69	72	75	68 |	94	113	127	136	143	149	153	157				

* Blood sugar values taken from curves which were constructed from values taken at staggered time intervals which varied in the different animals and are expressed in mg. per cent. ↑—Beginning of glucagon infusion.
† S—Single injection of glucagon, C—Continuous infusion of glucagon.
‡ Experiment started 16 hours after last food and insulin (regular).
§ Experiment started 11 hours after last food and insulin (regular).
‖ Experiment started 4 hours after last food and insulin (regular).
¶ A—Femoral artery, V—Femoral vein.

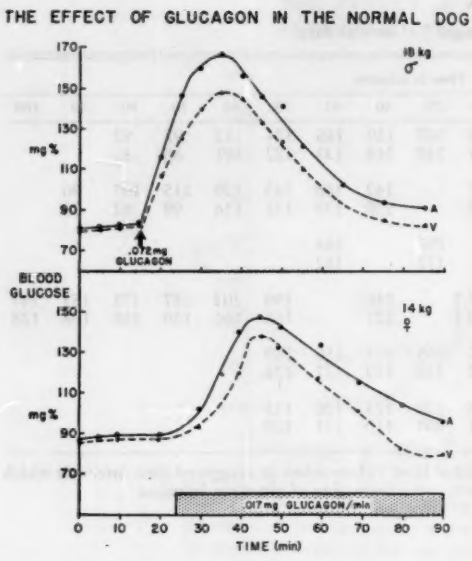

FIG. 3. THE EFFECT OF A SINGLE I.V. INJECTION OF GLUCAGON AND OF CONTINUOUS I.V. GLUCAGON INFUSION (IN NORMAL SALINE) ON (FEMORAL) ARTERIAL AND VENOUS GLUCOSE LEVELS IN THE NORMAL DOG

These observations are of considerable importance from the standpoint of glucagon's potential role in the regulation of carbohydrate metabolism. They indicate that glucagon has two integrated actions; namely, the mobilization of liver glycogen and the enhancement of the peripheral utilization of glucose. The persistence of the peripheral effect beyond the disappearance of the hyperglycemia suggests that glucagon has a more prolonged action than hitherto believed. The occurrence of these two effects of glucagon in preliminary experiments in the depancreatized dog indicates that the effects are independent of insulin action.

There is some experimental evidence which suggests that pituitary growth hormone causes the release of glucagon and that part of the diabetogenic action of the former is due to the released glucagon (1, 2). This hypothesis is not supported by the present experiments which show that glucagon increases the peripheral utilization of glucose; whereas there is abundant evidence that growth hormone decreases carbohydrate utilization (26).

The lack of change in Na^{22} space with glucagon

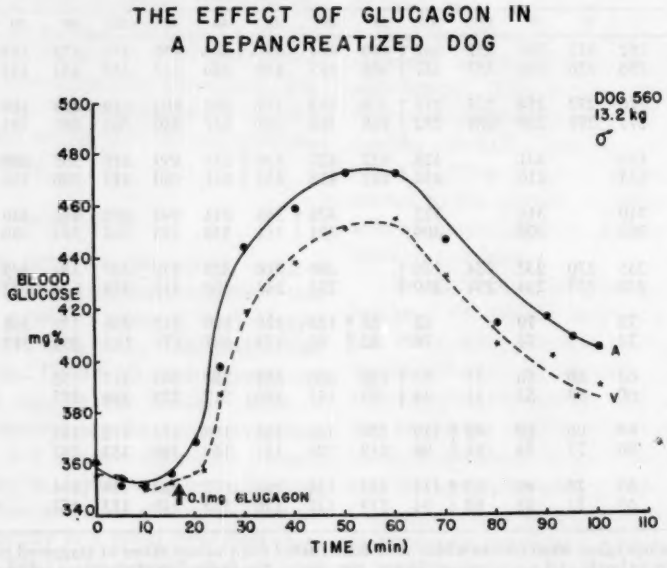

FIG. 4. THE EFFECT OF A SINGLE I.V. INJECTION OF GLUCAGON ON (FEMORAL) ARTERIAL AND VENOUS GLUCOSE LEVELS IN THE DEPANCREATIZED DOG

Experiment was done 16 hours after the last dose of insulin and food.

or glucose infusions at two different infusion rates indicates that no significant alteration in extracellular space has taken place during the experiments.

SUMMARY AND CONCLUSIONS

Studies on the effect of glucagon on blood glucose utilization in normal men using a constant glucose infusion technique have been presented. They show that glucagon causes a highly significant increase in the peripheral utilization of glucose which persists after the disappearance of the hyperglycemia. This increased glucose utilization is significantly greater than that observed with the hyperglycemia produced by glucose alone. The findings suggest that glucagon may fulfil a dual role in carbohydrate metabolism by producing hyperglycemia through mobilization of liver glycogen and concomitantly increasing the peripheral utilization of blood glucose. They also indicate that glucagon has a more prolonged action than previously thought.

Preliminary studies in normal and depancreatized dogs are also reported. Both groups of animals respond to glucagon in the same way as the normal human subject. These experiments indicate that the enhancing action of glucagon on the peripheral utilization of glucose does not depend upon a release of insulin.

APPENDIX

In the normal dog (18) it has been shown that the peripheral utilization of glucose (P) is a linear function of arterial glucose concentration (A). Therefore:

$$P \propto A$$

or

$$P = kA, \qquad (1)$$

where k is the proportionality constant. At any time t:

$$P = R\,(A - V), \qquad (2)$$

where R is the blood flow and V is the venous blood glucose concentration. If (*1*) and (*2*) are equated, it may be shown that:

$$\frac{A - V}{A} = K \text{ (constant)}, \qquad (3)$$

where K is a function of the specific rate constant of peripheral glucose utilization, the volume of glucose distribution and peripheral blood flow. If blood flow and glucose distribution remain constant, then $A - V/A$ must remain constant unless the rate constant of peripheral glucose utilization changes.

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STUDIES ON THE EPIDEMIOLOGY OF SARCOIDOSIS IN THE UNITED STATES: THE RELATIONSHIP TO SOIL AREAS AND TO URBAN–RURAL RESIDENCE

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During World War II, there were 350 recognized cases of sarcoidosis among American Armed Forces personnel. These cases, chosen in a relatively unselected manner, afford an admirable opportunity for investigation into some of the aspects of the epidemiology of this disease. Preliminary studies of this group have demonstrated a striking concentration of patients' birthplaces in the Southeastern United States (1, 2). Although cases in negroes predominated, the observed geographic localization could not be accounted for solely by the distribution of the negro population in the United States. The present investigation, which was concerned with factors which might have influenced this unusual case distribution, revealed an association between the distribution of the cases and certain soil areas. Furthermore, a higher incidence of the disease was found among servicemen from the more rural areas of the Southeast. These correlations may serve as additional localizing factors for further studies on the epidemiology of sarcoidosis.

MATERIAL AND METHODS

Of the 350 cases of sarcoidosis diagnosed in World War II servicemen, there were 334 living at the time of this study. Only these were investigated since the compilation of epidemiologic data would be incomplete in those deceased. In many patients the disease was discovered accidentally by routine x-ray examination taken during the course of hospitalization or upon separation from service. The diagnoses of sarcoidosis were made in American military hospitals scattered over the world. In all instances the biopsy specimens were reviewed and confirmed by the Armed Forces Institute of Pathology. A record of the induction chest film and physical examination was available on all patients. The complete records of all hospitalizations on each patient were studied and an analysis of the clinical data is in preparation. The standards for verification of the diagnosis have been previously reported (1, 2). The present analysis has been restricted to 297 of these cases, 37 were excluded because of the lack of availability of data to clearly establish locale of residence or diagnosis.

The minimum information on these cases consisted of identifying data including race, birthplace, residence at the time of entry into the military service, *i.e.*, "induction residence", and clinical and pathological reports which conformed to the criteria for the diagnosis of the disease, as proposed by the National Research Council (1). Additional consideration was given to those cases whose birthplace and induction residence were in the same or adjacent counties. For convenience, these cases have been arbitrarily designated "lifetime residents".

Figures giving the total number of persons serving in all the military services from the beginning of conscription to December 2, 1946, were obtained through the courtesy of the National Selective Service. These data, available by county and state of induction and race, were used as a basis for calculating race specific attack rates. Since the numerator consists of servicemen, a denominator representing the source of this group would be needed for calculation, hence the use of this Selective Service data. While certain factors of bias, such as omission of females, older persons and those rejected from military duty, are recognized in these rates, this military service information represented the most appropriate denominator that was available. The rates by induction residence, in turn, were selected because factors of migration, particularly the movement of Negroes from the Southeast to the northern industrial centers, prolonged the determination of accurate rates by birthplace.

The soil classification data which were used in this study were obtained through the courtesy of the Division of Soil Survey, United States Department of Agriculture. By the use of a recent map, showing the soil regions of the South, the various counties in Alabama,

[1] Present address: District State Health Officer, District Office, 400 Foote Bldg., 316 So. Warren St., Syracuse, New York.

[2] Present address: Sinai Hospital of Baltimore, Baltimore, Maryland.

[3] Present address: Maimonides Hospital of Brooklyn, 4802 Tenth Avenue, Brooklyn 19, New York.

Arkansas, Florida, Georgia, Louisiana, Mississippi, North Carolina, South Carolina, and Texas were placed in one of 91 categories, depending upon the predominant soil series charted for each county. The soil series, in turn, were combined into larger groups on the basis of similarities in topography, profile, and parent materials, to form the soil areas characteristic of the Piedmont Plateau, Middle and Lower Coastal Plain, and other physiographic divisions of the Southeast. These soils are contained for the greater part in 8 of the 36 great soil groups thus far recognized in the United States, namely, the Red-Yellow Podzolic soils, Rendzinas, Alluvial soils,

TABLE I

Cases of sarcoidosis in the Armed Forces during World War II, according to states, by race, birthplace, and induction residence

State	Number of cases					
	Birthplace			Induction residence		
	Total	White	Negro	Total	White	Negro
Region I (South Atlantic Gulf Coast)						
Alabama	31	5	26	27	5	22
Arkansas	13	4	9	14	3	11
Florida	11	1	10	15	—	15
Georgia	26	3	23	20	3	17
Louisiana	13	2	11	12	2	10
Mississippi	22	3	19	19	2	17
North Carolina	36	4	32	29	4	25
South Carolina	30	2	28	15	2	13
Texas	23	4	19	20	6	14
Virginia	18	2	16	14	2	12
Total	223	30	193	185	29	156
Region II (Middle Atlantic and Border)						
Delaware	1	—	1	1	—	1
District of Columbia	—	—	—	6	—	6
Maryland	3	—	3	4	—	4
New Jersey	2	2	—	7	1	6
New York	13	12	1	18	15	3
Oklahoma	3	2	1	3	1	2
Pennsylvania	3	3	—	14	4	10
Tennessee	8	4	4	8	3	5
West Virginia	3	2	1	2	1	1
Total	36	25	11	63	25	38
Region III (Rest of Country)*						
Arizona	—	—	—	1	—	1
California	1	—	1	6	4	2
Connecticut	—	—	—	1	1	—
Illinois	5	3	2	8	5	3
Indiana	3	3	—	4	3	1
Iowa	2	2	—	—	—	—
Kansas	1	—	1	2	1	1
Kentucky	1	1	—	1	1	—
Massachusetts	4	4	—	4	4	—
Michigan	2	1	1	8	3	5
Missouri	2	1	1	3	2	1
Montana	1	1	—	1	1	—
Nebraska	1	1	—	1	1	—
North Dakota	1	1	—	—	—	—
Ohio	3	1	2	6	2	4
Utah	1	1	—	1	1	—
Washington	2	2	—	2	2	—
Wisconsin	1	1	—	—	—	—
Total	31	23	8	49	31	18
U. S. Total	290†	78	212	297	84	212

* No cases were recognized from 11 states: Colorado, Idaho, Maine, Minnesota, Nevada, New Hampshire, Oregon, Rhode Island, South Dakota, Vermont, Wyoming.

† Seven white cases were foreign born.

TABLE II

Sarcoidosis attack rates among World War II servicemen, by race and induction residence, according to regions of the United States

	Selective service population			Sarcoidosis cases by induction residence			Attack rates per 100,000			Ratio N/W
	Total	White	Negro	Total	White	Negro	Total	White	Negro	
Region I (South Atlantic Gulf Coast)	2,955,135	2,291,056	664,079	185	29	156	6.3	1.3	23.5	18/1
Region II (Middle Atlantic Border)	4,238,992	3,953,837	285,155	63	25	38	1.5	0.6	13.3	22/1
Region III (Rest of U. S.)	7,037,448	6,820,041	217,407	49	31	18	0.7	0.5	8.3	17/1
Total	14,231,575	13,064,934	1,166,641	297	85	212	2.1	0.7	18.2	26/1
Ratio of Rates: Region I/Region III							9/1	3/1	3/1	
Region I/Region II							4/1	2/1	2/1	

Reddish-Chestnut soils, Ground-Water Podzolic soils, and Lithosols.[4]

The urban-rural distribution data which were used in this study were obtained from the 1940 census report. The population characteristics utilized in the urban-rural classification included population density, as defined by population per square mile, and total population. In relation to the negro cases, the population of negro inhabitants of a county classified as rural by the Bureau of Census was used. For this part of the study a total of 185 cases was studied. Each of these patients had entered military service from one of the ten Southeastern states, the area in which the highest incidence of the disease was found. An attempt was made to characterize the county areas of residence as predominantly rural or urban on a relative basis and to consider attack rates among servicemen from county areas of varying urban-rural composition.

RESULTS

Geographic distribution

The breakdown of cases by states is shown in Table I. The states have been divided into three geographic areas on the basis of the relative incidence of the disease. Region I, which had the highest incidence of sarcoidosis, was composed of the nine South Atlantic and Gulf Coast states of Alabama, Florida, Georgia, Louisiana, Mississippi, North Carolina, South Carolina, Texas, and Virginia plus Arkansas. In these states there was a total of 223 cases by birthplace, 30 whites and 193 negroes; and 185 by induction, 29 whites and 156 negroes. The difference of 37 negroes indicates the migration of this group out of the section. Region II which had the next highest incidence was contiguous to Region I and comprised the District of Columbia, the five Middle Atlantic States of Delaware, Maryland, New Jersey, New York and Pennsylvania, plus Tennessee, West Virginia and Oklahoma. In these states there was a total of 36 cases by birthplace, 25 whites and 11 negroes; and 63 by induction, 25 whites and 38 negroes. The difference of 27 negroes represents the migration into this industrial region. In the remainder of the country, Region III, there were 31 cases by birthplace, 23 whites and 8 negroes; and 49 by induction, 31 whites and 18 negroes.

Attack rates for sarcoidosis, specific for race and induction residence, are summarized in Table II for the three regions. In the South Atlantic Gulf Coast States, Region I, the overall attack rates are 1.3 and 23.5 for whites and negroes, respectively. The overall rate in the Middle Atlantic and Border States, Region II, was 1.5 per 100,000, 0.6 for whites and 13.3 for negroes. In Region III, the remainder of the country, the overall rate was 0.7 per 100,000, 0.5 among whites and 8.3 among negroes. It is apparent from these attack rates that a marked predominance of the disease exists among negro inductees of all regions, with the remarkably consistent negro-to-white ratios of 18 to 1, 22 to 1, and 17 to 1 for Regions I, II, and III, respectively. It is possible, however, that the disproportionately larger num-

[4] The classification and nomenclature of soils used in this study is that of Soils and Men, U. S. Dept. of Agr. Yearbook of Agriculture, 1938, Part IV, pp. 861–1161, with some modifications outlined in the issue of Soil Science and Soil Classification, Vol. 67, No. 2, 1949.

TABLE III

Sarcoidosis attack rates among World War II servicemen, by race and induction residence, according to the states of the South Atlantic Gulf Coast region

State	Selective service population			Attack rates per 100,000			$\frac{(O-E)^2}{E}$ (for Negro cases)
	Total	White	Negro	Total	White	Negro	
Alabama	272,843	202,648	70,195	9.9	2.5	31.3	1.83
Arkansas	189,327	156,550	32,777	7.4	1.9	33.6	1.41
Florida	208,787	157,599	51,188	7.2	0	29.3	0.75
Georgia	292,172	220,912	71,260	6.8	1.4	23.9	0.01
Louisiana	242,943	170,620	72,323	4.9	1.2	13.8	2.88
Mississippi	211,667	125,592	86,075	9.0	1.6	19.8	0.51
North Carolina	349,809	276,260	73,549	8.9	1.4	34.0	3.43
South Carolina	179,664	125,201	54,463	8.3	1.6	23.9	0.00
Texas	716,250	626,258	89,992	2.8	1.0	15.6	2.39
Virginia	291,673	229,416	62,257	4.8	0.9	19.3	0.46
Total	2,955,135	2,291,056	664,079	6.3	1.3	23.5	$X^2 = 13.67$
							$0.20 > p > 0.10$

ber of negro, as compared to white, emigrants from the Southeast may have altered the negro-to-white attack rate ratios for Regions II and III.

In comparing rates between the regions, it can be seen that the overall rate for Region I, 6.3 per 100,000, is nine times as great as that for Region III, 0.7 per 100,000. However, a large part of this difference is due to the disproportionate rates between the two races, since a ratio of less than 3 to 1 is observed between the respective

FIG. 1. THE DISTRIBUTION, BY BIRTHPLACE, OF 290 CASES OF SARCOIDOSIS RECOGNIZED AMONG WORLD WAR II SERVICEMEN

Each dot represents the birthplace of a single case. Seven additional cases were foreign-born.

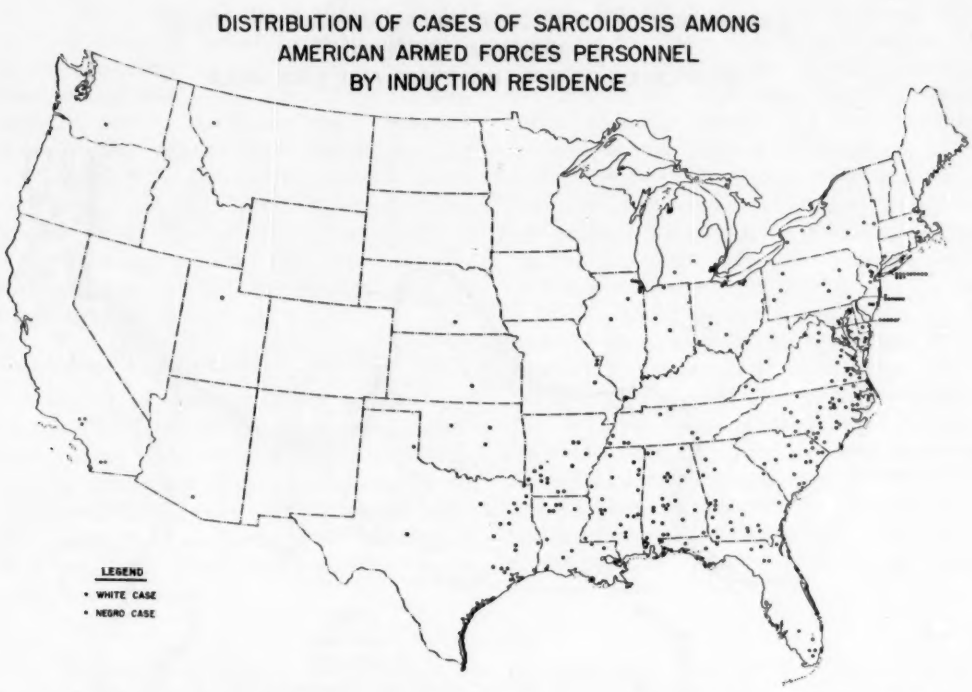

FIG. 2. THE DISTRIBUTION, BY RESIDENCE AT THE TIME OF INDUCTION INTO THE ARMED FORCES, OF 297 CASES OF SARCOIDOSIS RECOGNIZED AMONG WORLD WAR II SERVICEMEN

Each dot represents the induction residence of a single case.

negro and white rates for these regions. Similarly, the 4 to 1 ratio between the overall rates of Regions I and II, 6.3 and 1.5, respectively, also reflects the disproportionate rates between the negroes and whites.

It is evident, therefore, that the higher attack rate for sarcoidosis in the negro and the large negro population in Region I account, in part, for the concentration of cases in this area. However, the 3 to 1 ratio of rates in negroes for Regions I and III clearly indicates the presence of some localizing factor, aside from race, which influences the distribution of the cases.

In attempting to disclose what this factor (or factors) might be, subsequent considerations were focused on the ten states of Region I. Attack rates specific for race and induction residence were first determined for each of these states, as shown in Table III. Because of the small number of white cases, comparisons were made between the negroes only. In the table, the observed incidence of the disease among negro inductees for each state has been compared with the expected incidence on the basis of the overall rates for the area. It is evident that the variations obtained are not great. Although the negro attack rate for North Carolina, 34.0 per 100,000, is somewhat greater than that for the rest of the area, 22.2 per 100,000, this difference is only of questionable statistical significance. Thus, when comparing individual states as units in this manner, it is apparent that no definite concentration of the cases by induction residence exists in any one state.

Relation of the disease to the fall line

Reference to Figures 1 and 2 will reveal certain peculiarities in the distribution of the cases in the Southeast, both by birthplace and induction residence. Within the Carolinas, a greater number of residences appear in the eastern portion of the states. Concentrations also appear in Southern

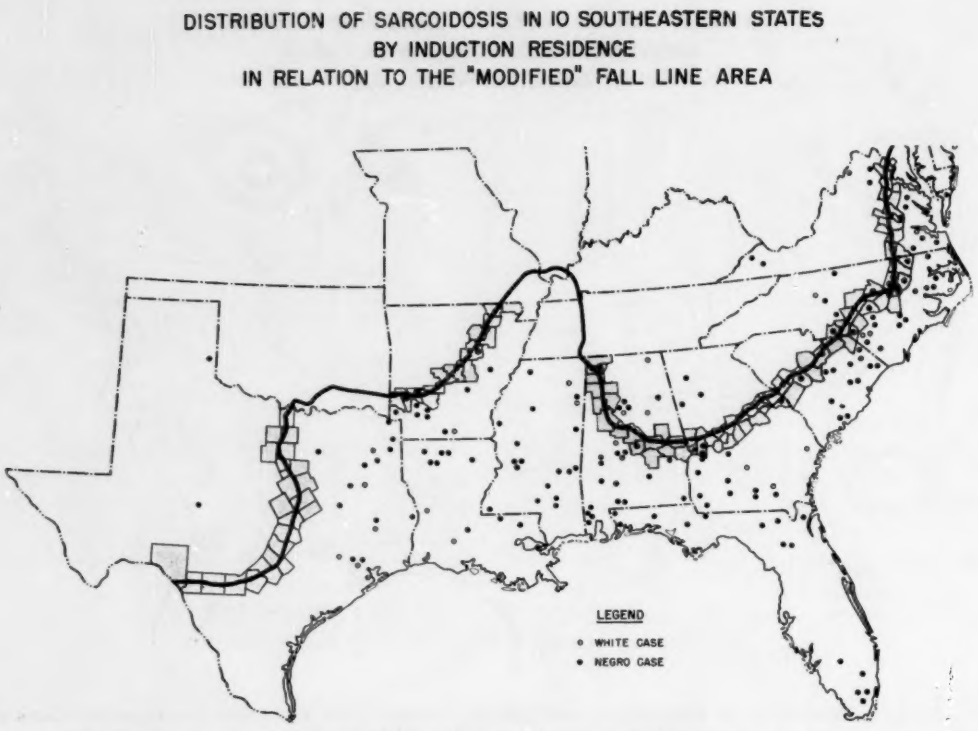

FIG. 3. THE DISTRIBUTION, BY INDUCTION RESIDENCE, OF 185 CASES OF SARCOIDOSIS IN THE 10 SOUTH ATLANTIC GULF COAST STATES OF REGION I IN RELATION TO THE FALL LINE AND "BORDER COUNTIES"

Each dot represents the induction residence of a single case. The heavy line represents the approximate location of the fall line and the shaded area indicates the "border" counties.

Georgia and in the area where Texas, Louisiana, and Arkansas are contiguous. This distribution corresponds very closely with the physiographic area known as the Atlantic and Gulf Coastal Plain. Until relatively recent geologic times, this area was covered by the sea and was delineated along its westernmost and northernmost extent by the former coast line, which is now known as the fall line (3). The approximate position of this former coast line is shown by the heavy line on Figure 3.

Comparisons were next made of the race specific attack rates by induction residence for the areas within the Southeastern states in relation to this geologic landmark. In arriving at these rates, counties were assigned to the "above" or "below fall line" category, depending upon their localization. Since the former coast line transects many contemporary county borders, and since the determination of its exact location in any region may be difficult, the counties traversed by and adjacent to the fall line, which have been designated as border counties, were considered separately. The term "modified" fall line was introduced to represent the composite area of these border counties and is depicted in Figure 3 by the stippled area.

Table IV indicates *negro* attack rates by induction residence for the areas of the ten states of Region I in relation to the "modified" fall line. It can be seen that for each state a greater number of cases in negroes occurs in the "below fall line" area. Similarly, the attack rate is greater for this area in each of the states, with the exception of Texas. The overall rate for the "below fall line" area in four of the states, Alabama, Georgia, North Carolina, and South Carolina, is 49.0 per 100,000 or five times greater than the

overall "above fall line" rate of 10.0 per 100,000 for these states. In three other states, Arkansas, Texas, and Virginia, the differences in attack rates between the "above" and "below fall line" areas are not statistically significant. However, in two of these, Arkansas and Virginia, the trend of the rates is the same as that observed in the four states where the differences are significant. Thus, we have a distinct intrastate concentration of the cases in the coastal plain area which appears to be unaccountable for by population distribution within these states.

Relation to other geographic factors

A number of ecological factors were explored for a possible explanation of this unusual localization. Among these were meteorologic conditions (4), the distribution of plant and animal life (5, 6), agricultural practices (7), and overall population densities (8). It was found that none of these factors was overtly associated with the distribution of the cases.

Since the fall line delineates, among other things, the boundaries of geologic formations (9) and major soil groups in the Southeast, attention was directed to a consideration, in a qualitative fashion, of surface materials. A close association appeared to exist between the residences of the cases and some of the soils of the coastal plains. This correlation was strengthened by several additional observations. In the Mississippi Valley, relatively few cases were noted although this is an area with a large negro population. The Alluvial soils, which are found there, differ significantly in character from the other soils of the Southeast. Furthermore, in the area where Texas, Louisiana, and Arkansas are contiguous, there is a reappearance of some of the soils which are present along the eastern portion of the coastal plain. The concentration of cases in this general region appeared to strengthen the preliminary correlation of the cases with these soils. Therefore, a more detailed and quantitative study was made of this relationship.

TABLE IV

Sarcoidosis attack rates among World War II Negro servicemen, by induction residence, according to areas in relation to the "modified" fall line in the South Atlantic Gulf Coast states

State	Area in relation to "modified" fall line	Negro selective service population	Negro cases by induction residences	Attack rates per 100,000	X^2 of differences between Negro rates
Alabama	Above	28,751	4	13.9	7.66
	Below	25,760	15	58.2	
Arkansas	Above	2,142	—	—	0.46*
	Below	23,543	8	34.0	
Florida	Below	51,188	15	29.3	—
Georgia	Above	30,721	3	9.8	5.81
	Below	26,636	11	41.3	
Louisiana	Below	72,323	10	13.8	—
Mississippi	Below	86,075	17	19.8	—
North Carolina	Above	33,498	3	9.0	10.38
	Below	21,745	12	55.2	
South Carolina	Above	17,237	—	—	5.67*
	Below	26,214	11	42.0	
Texas	Above	5,318	2	37.6	1.30
	Below	74,301	12	16.2	
Virginia	Above	22,101	4	18.1	0.21
	Below	24,767	6	24.2	
Total	Above	139,768	16	11.4	11.07
	Below	432,552	117	27.0	
	Border	91,759	23	25.1	

* Corrected for continuity.

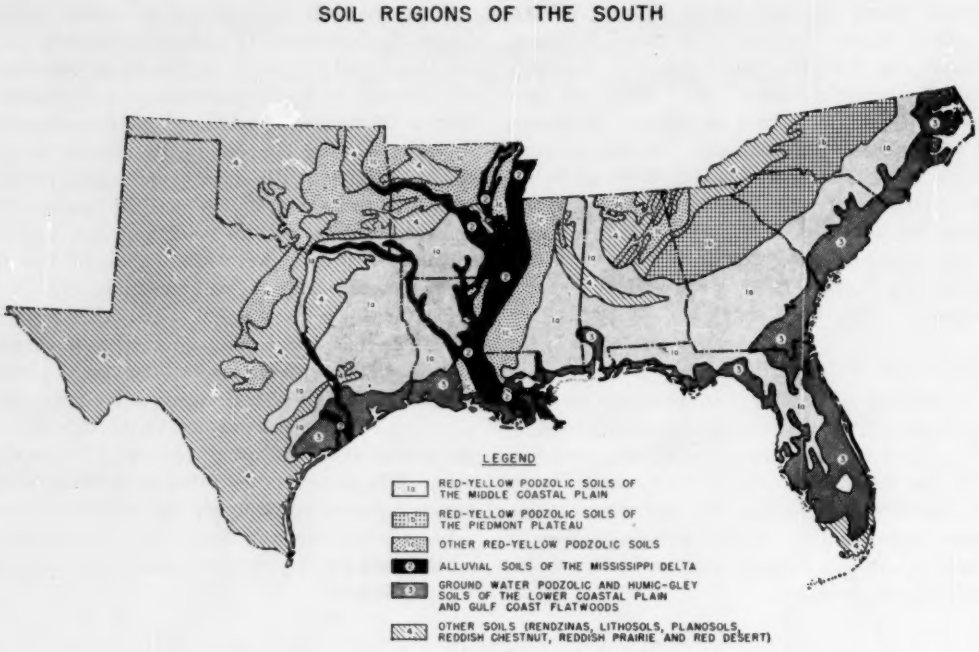
SOIL REGIONS OF THE SOUTH
LEGEND
RED-YELLOW PODZOLIC SOILS OF THE MIDDLE COASTAL PLAIN
RED-YELLOW PODZOLIC SOILS OF THE PIEDMONT PLATEAU
OTHER RED-YELLOW PODZOLIC SOILS
ALLUVIAL SOILS OF THE MISSISSIPPI DELTA
GROUND WATER PODZOLIC AND HUMIC-GLEY SOILS OF THE LOWER COASTAL PLAIN AND GULF COAST FLATWOODS
OTHER SOILS (RENDZINAS, LITHOSOLS, PLANOSOLS, REDDISH CHESTNUT, REDDISH PRAIRIE AND RED DESERT)

FIG. 4. THE GENERAL PATTERN OF THE GREAT SOIL GROUPS IN THE SOUTHEAST

Local details have been submerged in order to present a general view of the significant features on a small map.

Soils of the southeast

The general pattern of the soil areas in the Southeast is shown in Figure 4.[5] These soils can be differentiated on the basis of well-defined morphological and other characteristics, such as chemical composition, geologic precursors, native vegetation, and topography (10, 11).

The Red-Yellow Podzolic soils form the dominant group in this region, extending from Virginia to Texas. These have light-colored sandy surfaces overlying firm, red to yellow subsoils. They are generally well drained, strongly leached, and low in organic and mineral plant nutrients.

On the basis of variations in one or more of their characteristics, these leached soils can be further classified among a number of subgroups. In Figure 4 are outlined The Red-Yellow Podzolic soils of the Middle Atlantic Coastal Plain, subgroup "1a," those of the Piedmont Plateau, subgroup "1b," and a miscellaneous subgroup, "1c."

The Alluvial soils occupy the flood plains in the Southeast and form the dominant soils of the Mississippi Valley. They are soils in early stages of profile development, having been affected very little by processes of soil formation. In general, the Alluvial soils in this region are relatively high in organic matter, poorly drained, and neutral in reaction. These have been designated as group "2" in Figure 4.

The Humic-Grey and Ground-Water Podzols are poorly drained soils which occur extensively along the Atlantic and Gulf Coasts. These soils, group "3" in Figure 4, generally have light-grey sandy leached layers and usually contain little organic matter. Most of these have been derived from beds of unconsolidated sands and sandy clays of relatively recent geological origin.

The remaining soils in the Southeast are com-

[5] Since identical soil classification data were not available for Virginia, the subsequent analysis has been restricted to nine of the states in Region I. Older soil classification schemes (10) indicated that the inclusion of Virginia did not significantly alter the correlations obtained between race specific attack rates for sarcoidosis and residence in certain soil areas.

TABLE V

Sarcoidosis attack rates among World War II Negro servicemen, by induction residence, according to soil areas of the South Atlantic Gulf Coast states *

Soil group	Negro selective service population	Negro cases by induction residence	Attack rates per 100,000	$\frac{(O-E)^2}{E}$ (for Negro cases)
Red-Yellow Podzolic soils of the Middle Coastal Plain (1a)	188,049	82	43.6	30.65
Red-Yellow Podzolic soils of the Piedmont Plateau (1b)	92,542	6	6.5	11.72
Other Red-Yellow Podzolic soils (1c)	63,995	11	17.2	1.21
Alluvial soils of the Mississippi Delta (2)	55,881	6	10.7	4.09
Ground-Water Podzolic and Humic-Clay soils of the Lower Coastal Plain (3)	116,376	23	19.8	0.83
Other soils† (4)	84,979	16	18.8	0.91
				$X^2 = 49.41$
				$p < 0.001$
Totals	601,882	144	23.9	

* Includes Alabama, Arkansas, Florida, Georgia, Louisiana, Mississippi, North Carolina, South Carolina, Texas.
† Includes Rendzinas, Lithosols, Plenosols, Reddish Chestnut, Reddish Prairie and Red Desert soils.

posed of a heterogeneous group of shallow, stony soils (Lithosols), clay pan soils (Planosols), black soils (Rendzinas), and subhumid and semi-arid soils (Reddish-Prairie and Reddish-Chestnut), all of which have been combined into group "4" in Figure 4.

Negro attack rates by soils

Race specific attack rates for negroes by induction residence were determined for the various soil areas in the Southeast, as shown in Table V. Although the number of white cases in relation to each soil group was too small to make valid comparisons of the attack rates between the groups, the trends observed were similar to those among the negro patients.

The negro attack rates range from 6.5 per 100,000 for the soils of the Piedmont Plateau above the fall line to 43.6 per 100,000 for the soils of the Middle Coastal Plain below the fall line. The attack rate for the Alluvial soil area is somewhat low, 10.7, but the remaining rates appear to be quite uniform. Thus, rates of 17.2, 19.8, and 18.8 per 100,000 were noted for the group "1c," group "3," and group "4" soil areas, respectively.

It is apparent that the greatest differences in attack rates exist between the soils of the Middle Coastal Plain and those of the Piedmont Plateau, where a ratio of almost 7 to 1 in these rates is observed. This ratio should be contrasted to the much smaller 3 to 1 ratio which was found between the negro attack rates for Regions I and III.

No significant localization of the cases by induction residence appeared to exist within the Red-Yellow Podzolic soils of the Middle Coastal Plain itself. Thus, negro attack rates in relation to this particular soil group for each of the nine Southeastern states, as shown in Table VI, are essentially equivalent. The one exception, perhaps, is North Carolina, where the rate of 71.3 per 100,000 is somewhat higher than those for the other states. Even with this elevated rate, however, the total chi square of the differences, 10.48, is not statistically significant.

TABLE VI

Sarcoidosis attack rates among World War II Negro servicemen from the "1a" soil area, according to states

State	Negro selective service population	Negro cases by induction residence	Attack rates per 100,000	$\frac{(O-E)^2}{E}$ (for Negro cases)
Alabama	27,175	13	47.8	0.12
Arkansas	10,464	7	66.9	1.25
Florida	10,001	6	60.0	0.58
Georgia	27,059	13	48.0	0.12
Louisiana	18,574	5	26.9	1.19
Mississippi	20,898	8	38.3	0.13
North Carolina	22,449	16	71.3	3.92
South Carolina	19,374	5	25.8	1.38
Texas	32,055	9	28.1	1.79
				$X^2 = 10.48$
				$0.20 < p < 0.30$
Totals	188,049	82	43.6	

Race specific attack rates were determined for each of the seventeen minor soil series which compose the leached soils of the Middle Coastal Plain. Because of the generally small number of cases, it was difficult to compare the rates, which were found to be quite variable. However, no significant concentration of induction residences was observed in relation to any of these soil series, as can be observed from Figure 5. There, the approximate locations of the individual soil series are indicated by the alphabetical characters. Thus, the factor (or factors) which influence the concentration of cases by induction residence in the Middle Coastal Plain area appears to be distributed with some uniformity.

Factors of migration

Admittedly, these efforts to quantitate the association of the cases of sarcoidosis with certain soil groups are crude. However, the previously mentioned influence of migration, particularly for the negroes, seems to minimize the observed differences. For example, of the 52 negroes inducted from Regions II and III, only 17 were born in this area; 35 had been born in the Southeast. Of these 35 migrants, 22, or 63 per cent were born in the area of the Middle Coastal Plain soils. Since it is reasonable to assume that the majority of these individuals had migrated to northern areas as recently as the pre-war period of national mobilization, exposure to the soils may have been of considerable duration.

The uncertainties introduced by migration can be minimized by considering only those cases whose birthplace and induction residence were located in the same or adjacent counties, the so-called "lifetime residents." It would seem reasonable to assume that these individuals had a pro-

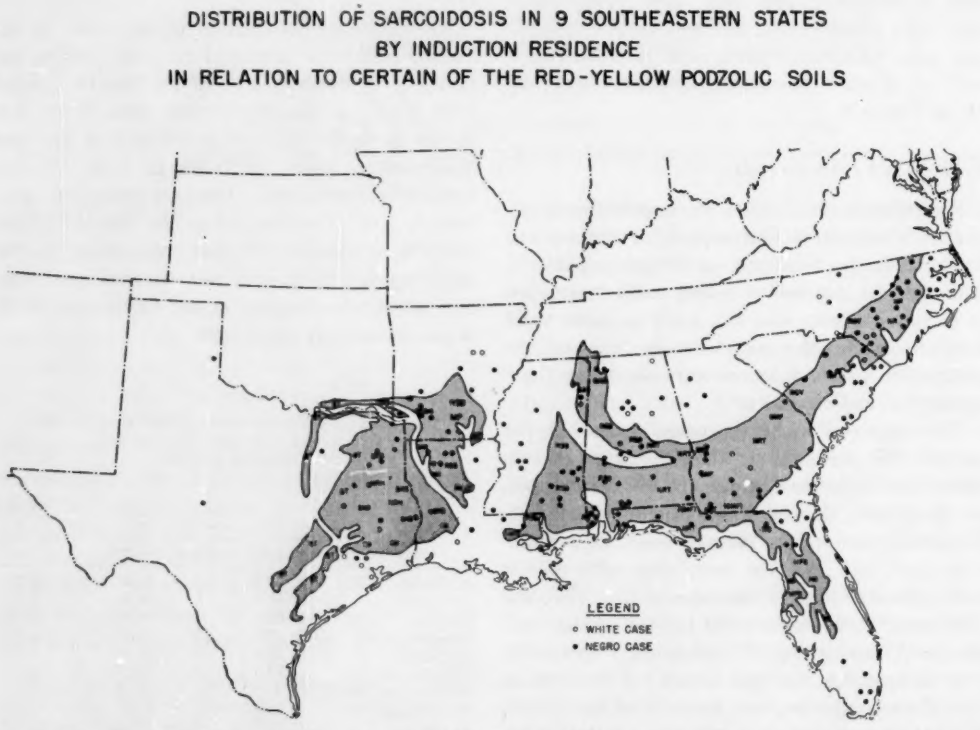
DISTRIBUTION OF SARCOIDOSIS IN 9 SOUTHEASTERN STATES
BY INDUCTION RESIDENCE
IN RELATION TO CERTAIN OF THE RED-YELLOW PODZOLIC SOILS
LEGEND
WHITE CASE
NEGRO CASE

FIG. 5. THE INDUCTION RESIDENCES OF A GROUP OF 171 CASES OF SARCOIDOSIS IN NINE OF THE SOUTH ATLANTIC GULF COAST STATES

The residences are shown in relation to the 17 minor soil series, indicated by the alphabetical characters which compose the "1a" Red-Yellow Podzolic soils.

longed contact with the soils in and about their county of induction, whereas a similar assumption would be less likely to apply in the case of the migrants. Of the 113 negroes with sarcoidosis inducted from Region I who could be classified as "lifetime residents," 73 came from the Middle Coastal Plain soil area and 4 from the leached soil area of the Piedmont Plateau. The total negro selective service populations for these areas, inclusive of immigrants and permanent residents, were approximately 188,000 and 93,000, respectively. While these figures are not deemed sufficiently comparable to calculate valid attack rates, the crude rates which may be derived are 39 per 100,000 and 4 per 100,000 indicating differences of 10 to 1 or greater. As noted previously, the attack rates based on the total number of cases inducted from these areas were 43.6 per 100,000 and 6.5 per 100,000, respectively, representing a difference of slightly less than 7 to 1.

Unfortunately, chronological residence data are not available for all the cases from Region I. If such were at hand, it would be feasible to determine man-years of experience for all the soil areas. The significant correlations obtained by use of "lifetime residents" suggest that such a method of analysis would strengthen the relationship between sarcoidosis and the soils of the Middle Atlantic Coastal Plain.

Urban-rural occurrence of the disease

By grouping the counties of the ten Southeastern states according to population density, sarcoidosis attack rates can be calculated for white and negro servicemen from these composite county areas. In Table VII the rates for negro military personnel are shown. It can be seen that an inverse relationship exists between these rates and county population density. For the counties with 19 or fewer inhabitants per square mile, presumably those which are most rural in character, the rate of 48.7 per 100,000 is highest. On the other hand, a much lower rate, 15.2 per 100,000, is found among the servicemen from the counties with population densities of 100 or more people per square mile, areas which presumably are most urbanized. The attack rates among negro servicemen from counties with intermediate population densities lie in between these extremes.

TABLE VII

*Sarcoidosis attack rates among World War II Negro servicemen from 10 South Atlantic Gulf Coast states * according to population density of county of induction*

Population density† of county of induction	Negro selective service population	Negro cases by induction residence	Attack rates per 100,000	$\frac{(O-E)^2}{E}$
Less than 20	20,560	10	48.6	5.63
20–49	219,332	71	32.4	7.38
50–99	187,434	39	20.8	0.57
100 and over	236,753	36	15.2	6.91
Total	664,079	156	23.5	$X^2 = 20.49$ $p < 0.001$

* Includes Alabama, Arkansas, Florida, Georgia, Louisiana, Mississippi, North Carolina, South Carolina, Texas, and Virginia.

† Population per square mile (1940), from County Data Book, U. S. Bureau of the Census, 1947.

The overall differences between these rates are statistically highly significant ($X^2 = 20.49$, $p < .001$). The small number of white cases in relation to each county group made it difficult to evaluate the differences between the attack rates observed. However, the overall trend of these rates was the same as that found among negro servicemen.

Figure 6 illustrates the population density distribution for the counties of the ten-state region. In comparing this with Figure 3, which illustrates the distribution of the cases of sarcoidosis in relation to the modified fall line, it appears that the more densely populated counties are located in the area of the Piedmont Plateau (above the fall line) whereas the distribution of the less densely populated counties appears to conform to that of the Atlantic Coastal Plain (below the Fall line).

In view of the previously demonstrated correlation between race specific attack rates for sarcoidosis and residence in certain soil areas in the Southeast, it was deemed desirable to determine whether urban-rural localizing factors were acting independently of these soil associations. Therefore, attack rates were computed for servicemen from the various soil areas of the Southeast according to the population density distribution of the various counties.

These rates for negro servicemen from the Red-Yellow Podzolic soil area of the Middle Atlantic Coastal Plain in nine of the Southeastern states having identical soil classification data are

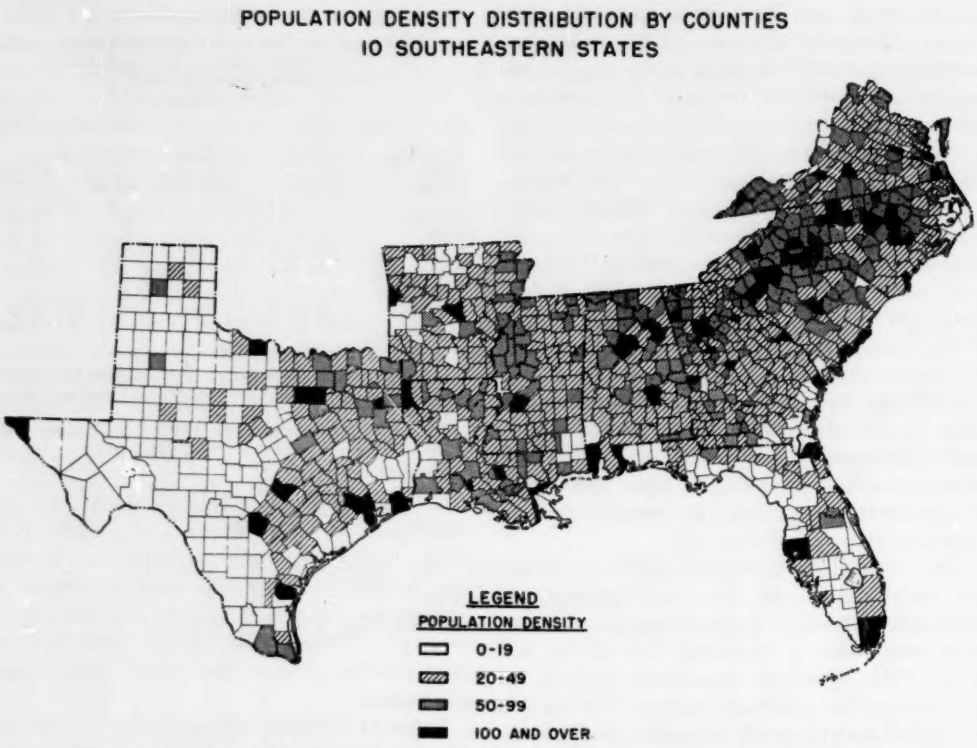

FIG. 6. THE POPULATION DENSITY DISTRIBUTION BY COUNTIES IN TEN SOUTHEASTERN STATES

Population density is expressed as number of inhabitants per square mile and refers to Bureau of the Census data from the 1940 census.

shown in Table VIII. This "1a" soil area, as designated previously, represents the site of maximal intraregional concentration of the cases, both by birthplace and induction residence. The rate of 91.8 per 100,000 among the servicemen from the counties with lowest population densities, 19 or fewer inhabitants per square mile, is more than twice as great as the overall rate of 41.7 per 100,000 among servicemen from the more densely populated counties. Although the number of cases in the former category is small, the difference between these rates is statistically significant ($p < 0.04$). The remaining attack rates among servicemen from counties with population densities of 20–49, 50–99, and 100 or more inhabitants per square mile, show no consistent downward trend, in contrast to the findings for the whole of the ten-state region.

On the other hand, the attack rates among negro servicemen from the remaining soil areas of the nine-state region exclusive of the "1a" Middle Coastal Plain area are shown in Table IX.

TABLE VIII

Sarcoidosis attack rates among World War II Negro servicemen from the Middle Coastal Plain soil area, by induction residence, according to population density of county of induction*

Population density† of county of induction	Negro selective service population	Negro cases by induction residence	Attack rates per 100,000
Less than 20	7,639	7	91.6
20–49	93,612	37	39.5
50–99	56,000	25	44.6
100 and over	30,798	13	42.2
Total	188,049	82	43.6

* Middle Coastal Plain Soil area in 9 states, including Alabama, Arkansas, Florida, Georgia, Louisiana, Mississippi, North Carolina, South Carolina, and Texas.

† Population per square mile (1940), from County Data Book, U. S. Bureau of the Census, 1947.

TABLE IX

Sarcoidosis attack rates among World War II servicemen from nine South Atlantic Gulf Coast states, exclusive of the Middle Coastal Plain soil area, according to population density of county of induction*

Population density† of county of induction	Negro selective service population	Negro cases by induction residence	Attack rates per 100,000
Less than 20	12,640	3	25.7
20–49	108,294	27	24.9
50–99	114,060	11	9.6
100 and over	178,779	21	11.7
Total	413,773	62	15.0

* Includes Alabama, Arkansas, Florida, Georgia, Louisiana, Mississippi, North Carolina, South Carolina, and Texas.

† Population per square mile (1940) from County Data Book, U. S. Bureau of the Census (1947).

Rates of 23.7 and 24.9 per 100,000 were obtained for servicemen from counties with population densities of 19 or fewer, and 20 to 49 inhabitants per square mile, respectively. The overall rate for these composite county areas, 24.9 per 100,000, is statistically significantly higher ($p < 0.001$) than the overall rate of 10.9 per 100,000 observed among servicemen from counties with greater population densities, 50 to 99, and 100 or more inhabitants per square mile, respectively. Again, there is no consistent downward trend in the rates as shown in Table VII for the entire ten-state region, but the qualitative relationships remain unchanged, *i.e.*, higher attack rates prevail among servicemen from counties with low population densities, which presumably are most rural in character. These findings suggest the presence of a rural localizing factor in the occurrence of sarcoidosis which may be independent of any primary soil relationship.

By grouping the counties of the "1a" Middle Coastal Plain soil area according to population size, a similar correlation can be demonstrated, as shown in Table X. Here, a threefold difference is evident between the attack rate of 124.4 per 100,000 among negro servicemen from counties with the smallest total populations, less than 10,000 inhabitants, and the overall rate of 41.1 per 100,000 among servicemen from counties with the smallest total populations, less than 10,000 inhabitants, and the overall rate of 41.1 per 100,000 among servicemen from counties with more than 10,000 inhabitants. Although the former rate is based on only seven cases, the difference between these rates is statistically significant ($p < 0.01$). Again, no consistent downward trend in the rates is apparent among the more populous counties; however, the rate among servicemen from counties with populations of 100,000 or more, is only 25.7 per 100,000, a somewhat lower value than the others.

A further suggestion of the rural localization of sarcoidosis is provided by other Bureau of the Census data. Table XI presents the attack rates among negro servicemen from the Middle Atlantic Coastal Plain soil area according to the proportion of inhabitants classified as "rural," *i.e.*, those residents in communities with less than 2500 people, among the negro population of each county. A direct correlation exists between these rates and the percentage of "rural" negro inhabitants. Thus, among negro servicemen from counties where 90 to 100 per cent of the negroes are in the "rural" category, the attack rate of 54.7 per 100,000 is highest. This declines to a rate of 29.0 per 100,000 for the servicemen from counties with less than 49 per cent "rural" negro inhabitants. Although the differences between these rates are only of borderline statistical significance, the trend is in conformity with previous findings.

Admittedly, these attempts to demonstrate a rural localization of sarcoidosis among World War II servicemen are also crude. The factor of migration, among others, again introduces some uncertainty in the interpretation of the findings and the unavailability of residence data pertaining to

TABLE X

Sarcoidosis attack rates among World War II Negro servicemen from the Middle Coastal Plain soil area, by induction residence, according to population size of county of induction*

Population size† of county of induction	Negro selective service population	Negro cases by induction residence	Attack rates per 100,000
Less than 10,000	5,622	7	124.5
10,000–19,999	30,502	14	45.9
20,000–49,999	95,854	39	40.7
50,000–99,999	40,514	18	44.4
100,000 and over	15,557	4	25.7
Total	188,049	82	43.6

* Middle Coastal Plain soil area in nine states, including Alabama, Arkansas, Florida, Georgia, Louisiana, Mississippi, North Carolina, South Carolina, and Texas.

† 1940 population, from County Data Book, U. S. Bureau of the Census, 1947.

TABLE XI

Sarcoidosis attack rates among World War II Negro servicemen from the Middle Coastal Plain soil area, by induction residence, according to per cent of rural inhabitants among Negro population of county of induction*

Per cent of rural inhabitants among Negro population of county of induction†	Negro selective service population	Negro cases by induction residence	Attack rates per 100,000
90–100	58,546	32	57.7
70–89	63,367	26	41.0
50–69	31,697	14	44.2
Less than 50	34,439	10	29.0
Total	188,049	82	43.6

* Middle Coastal Plain soil area in Alabama, Arkansas, Florida, Georgia, Louisiana, Mississippi, North Carolina, South Carolina, and Texas.
† From 1940 Bureau Census Data.

the pre-induction period precludes any attempt to correct for this variable. The importance of the latter is evident from the findings on the follow-up studies of 40 cases in the ten-state Southeastern region. Sixteen, or 40 per cent of these patients, lived in a community with a population of 2500 or greater at the time of their induction into the armed forces, and by the criteria of the Bureau of the Census, could be termed "urban" inhabitants. However, chronological residence histories from these sixteen individuals revealed that eleven had spent more than half their lives on farms or had engaged in farming as an occupation prior to military service, and therefore must have had prolonged contact with rural environments. It would seem likely that similar residence information from more intensive follow-up studies of all the cases would serve to strengthen the relationship between the disease and previous rural residence.

DISCUSSION

An analysis of the residence data from this group of 297 military cases of sarcoidosis revealed a distinct concentration of their birthplaces and induction residence in the Southeast. Within this region, a significant correlation was observed between race specific attack rates and residence in areas defined by the distribution of certain specific soil groups. In addition, significantly higher attack rates were noted among military personnel from the Southeast whose residences at the time of entry into the armed forces were located in the relatively rural counties, as evidenced by low population density and small population size of these areas.

Since sarcoidosis is not a reportable disease, there is a paucity of morbidity data from which any comparison might be made with the cases which occur in the general population. Israel and Sones (12) have recently described their experiences with cases of sarcoidosis seen in Philadelphia. Of the 74 negroes in their group, 64 were born in the South, a distribution greatly disproportionate to the estimated percentage of southern-born negroes in that city.

Some data from a group of civilian cases of sarcoidosis in North Carolina (13) and South Carolina (14) provided an opportunity to compare the geographic localization of these cases with that observed among the military group. Of a total of 14 individuals suspected of having tuberculosis but later diagnosed as sarcoidosis, during 1949 and 1950 in South Carolina, 12 resided in the "below fall line" area. Nine of these resided within counties containing the leached soils of the Middle Coastal Plain as the predominant soil types. In North Carolina, 17 deaths were reported from Boeck's sarcoid from 1949 to 1951. Of the total, four resided in counties above the fall line, six lived in "border" counties and seven resided in the "below fall line" area. Of the 13 deaths in "border" and "below fall line" counties, nine had lived where the leached Middle Coastal Plain soils abounded. Thus, these data, while inappropriate for the computation of sarcoidosis attack rates for different areas, would appear to be consistent with the observations on the military cases inducted from these states.

On the other hand, in analyzing the birthplaces of 177 patients with sarcoidosis diagnosed at the Mayo Clinic from 1940 through 1951, Carr and Gage (15) did not find that the disease was more common in patients who came from the Southeastern part of the United States than in those who came from other areas. Apparently, such factors as age, sex, race, and severity of disease could not explain the difference in distribution from that which had been reported for the group of military cases (2). It should be pointed out, however, that the comparison between the geographic distribution of cases and the total population at the Mayo Clinic does not provide any precise information as to the relative incidence of the disease

among an unselected population in a given area. The series of Carr and Gage therefore is not strictly comparable to the military group.

The evidence that Boeck's sarcoid occurs with greater frequency among inhabitants of rural areas is in accord with the suggestion that soils may be determinants in the occurrence of the disease. Longcope and Freiman (16) in confirming an earlier observation of Lomholt (17), noted that sarcoidosis often affected farm laborers and those who live in country districts. Whitehead (18) and Cone (19) refer to similar observations. In relating calcareous crystalline material in soils to the pathogenesis of lesions characteristic of sarcoidosis, Refvem (20) noted ". . . that people living in the country are preferably attacked by Boeck's disease, especially farmers, gardners, *etc.*" Israel and Sones (12) reported that of ten cases of sarcoidosis in white persons seen in Philadelphia only one had been a life-long resident of that city. They noted that "Sarcoidosis would appear to be extremely rare among what is the largest group in this country, the native-born urban white."

The higher attack rates of sarcoidosis among the rural inhabitants of the Southeastern United States, its apparent predilection for negroes and for those living in certain soil areas presents a challenging and perplexing problem. If sarcoidosis is a manifestation of a single etiologic agent, the widespread occurrence of cases in this country would indicate that such an agent is not confined to the Southeast. However, the distinct aggregation of military cases in this area and the suggestion of a similar distribution among civilian cases leads to speculations which may point to further investigation.

It is conceivable that the observed geographic localization of the disease is the result of a) the presence of a highly favorable environment in areas within the Southeast for the growth, propagation, or transmission of an etiologic agent or agents, or b) an alteration in the susceptibility of the population of these areas which predisposes them to the action of some agent which elsewhere might possess a very low grade or altered pathogenecity.

The role of possible host factors in the localization of sarcoidosis is suggested by experiences with pellagra in this country. The concentration of the latter disease in the Southeast could be related largely to the socio-economic status and consequent dietary habits of the affected population, with contributory or secondary factors, such as infectious diseases, influencing its incidence at a particular time or place (21). Similarly, dietary peculiarities and the socio-economic status of the population in certain areas of the Southeast may enhance their resistance to the inciting factor of sarcoidosis; however, little information is available on this point.

The data pertaining to the unusually high incidence of sarcoidosis among negroes in this country also suggest an influence of host susceptibility. Of the total of 523 cases reported in the American literature up to 1952, 385, or 73.4 per cent, were of this race (16). The 26 to 1 ratio between the overall negro and white attack rates in the present series of cases emphasizes this racial predilection. In contrast are the reports from Europe and elsewhere which indicate a high frequency of sarcoidosis in the white race (22). Schönholzer found 67 cases of sarcoidosis among 516,879 Swiss army recruits subjected to mass radiographic examinations, an incidence of 13 per 100,000 (23). This rate compares much better with the overall attack rate in negroes of 18 per 100,000, than the white rate of 0.7 per 100,000 among American servicemen. Although these two series are not strictly comparable, the results suggest that factors other than inherent racial susceptibility may be influencing the higher prevalence of sarcoidosis among negroes in this country. Thus, sociologic and environmental factors, such as those suggested to account for the significantly higher incidence of venereal diseases among this group (24), may similarly contribute to a susceptibility for Boeck's sarcoid.

The possible concentration of an etiologic agent in some of the soils of the Southeast is brought to mind by the somewhat similar distribution of hookworm disease in the sandy areas of this region (25), and the greater prevalence of this parasitic infection among rural inhabitants (26). Furthermore, recent epidemiologic studies of various mycotic diseases, particularly histoplasmosis and coccidioidomycosis, indicate that their distribution is restricted to regions containing soils favorable for the growth and dissemination of the causative fungi (27–29). By analogy, it can be postulated that some helminth, bacterium, fungus, or virus

giving rise to sarcoidosis may find certain of the Red-Yellow Podzolic soils of the Southeast the best natural media in which to thrive and thus result in the described localization of the disease.

Other factors, such as the role of intermediate vectors, may account for the circumscribed distribution of Boeck's sarcoid. The Southeastern localization of typhus and malaria can be correlated with the distribution of the tropical rat flea (*Xenopsylla cheopis*) and certain species of mosquitoes (*Anopheles quadrimaculatus*) (30, 31). In these instances the concentration and migration of the rat, as well as climatic conditions (32, 33), and the presence of impounded water and other suitable breeding places (34), rather than any specific soil characteristics, appear to be the factors which confine these respective arthropods to this region. However, certain insects are restricted to specific soil areas, thereby localizing disease agents for which they are the natural vector. Acute conjunctivitis in the South and the distribution of the sand gnat, (*Hippelates pusio*), may be such a case in point (35, 36). Again by analogy, it is possible that some intermediate arthropod vector, in close association with certain soils, serves as an effective transmitter of the etiologic agent of sarcoidosis. However, the lack of any evidence to incriminate an intermediate vector in the transmission of the disease and the lack of any knowledge concerning an arthropod distribution which conforms to that observed for the military cases casts some doubt upon such an hypothesis.

The *plant and animal life* of the soil areas in which cases of sarcoidosis are concentrated bear scrutiny for a possible etiologic relationship to the disease. In addition, the *chemical differences* between the various soils of the Southeast might have some relation to the localization of Boeck's sarcoid.

In regard to the latter, the Red-Yellow Podzolic soils of the Piedmont Plateau (where relatively few cases of sarcoidosis resided) contain a strikingly lower percentage of silica and higher percentages of alumina and potash than do the soils of the middle Atlantic Coastal Plain (10). These differences and the resemblance of the pulmonary forms of sarcoidosis to some of the pneumoconioses suggest the possibility that soils may be directly implicated in the pathogenesis of Boeck's sarcoid. In support of this are the observations of Refvem, who demonstrated crystalline material, which he assumed to be derived from calcareous spars in the soil, in biopsy specimens of 4 of 100 patients with sarcoidosis (20). Ayres, Ober, and Hamilton are of the opinion that many of the strictly localized sarcoid lesions of the skin, following trauma, are actually a form of silicosis (37), and similar contentions have been made by other workers (38, 39). Experimentally, Gardner was able to reproduce in rabbits, guinea pigs, and rats, granulomatous lesions resembling those of sarcoidosis, following the administration of particles of silicon dioxide (40).

Suggestions have been made of a relationship between sarcoidosis and the chronic form of beryllium poisoning (41). Although there are many similarities in clinical features between the two, certain differences have been found (42). Furthermore, as Freiman has noted (43), the relatively recent industrial use of beryllium compounds makes these agents unlikely causes for any great numbers of cases of sarcoidosis. However, the inability of Dutra, Cholak, and Hubbard (44) to recover abnormal quantities of beryllium from some lesions of chronic berylliosis, illustrates the difficulty of evaluating possible relationships.

The possibility does exist that exposure to unusual amounts of *naturally-occurring* beryllium in the soils of the Southeast might have accounted for the aggregation of cases in the middle Atlantic Coastal Plain. Soils in this area were derived from the granitic rocks of the Appalachian Mountains which represent the major concentration of beryllium containing surface ore in the United States. Similarly, such beryllium containing rocks have their greatest concentration in Europe in the Scandinavian countries where sarcoidosis is quite prevalent. Analysis of soil samples obtained in close proximity to the lifetime residence of several of the military cases revealed, by the specific gravity flotation method, the presence of occasional small fragments of beryllium containing rock (44). Chemical and spectrographic analyses carried out by Hodge, Toribara, Steadman, Cholak, and Hubbard, confirmed the presence of beryllium in these samples, although not in any unusual concentrations (45, 46). However, the need for caution in relating sarcoidosis to berylliosis and other forms of pneumoconioses which

might result from any association with soils must be stressed, in view of the diversity of agents that can induce tissue reactions closely simulating the sarcoid granuloma.

Many hypotheses relating to factors of host susceptibility or to characteristics necessary for the propagation and transmission of an etiologic agent may be invoked to account for the observed concentration of cases in certain areas of the Southeast. Undoubtedly, other possibilities exist which have not been mentioned. Any working hypothesis should be in accord with the available epidemiologic knowledge of sarcoidosis in this country; namely, its apparent predilection for negroes, its concentration in certain soil areas, and its apparent greater relative frequency among rural inhabitants. The newly defined hyper-endemic sarcoidosis area in the Southeast would appear to warrant more intensive study in an effort to elucidate the many obscurities still surrounding the etiology and pathogenesis of this disease.

SUMMARY

An epidemiologic study of 350 cases of sarcoidosis in military personnel during World War II has indicated the occurrence of this disease with a significantly greater frequency among those whose birthplace is in the Southeastern United States. Residence in a rural area further favors development of the disease.

The ratio of negro to white cases of 18 to 1 accounts in part only for this geographic distribution. The occurrence of sarcoidosis is greater in patients living within certain regions of this endemic area. A close association between the distribution of certain soil types with these regions was noted. Thus, residence in rural areas of the Southeast, particularly of negroes, within regions of a fine sandy soil type appears to favor the development of sarcoidosis. The possible implications of these findings are discussed.

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THRESHOLD OF RESPONSE OF THE CEREBRAL VESSELS OF MAN TO INCREASE IN BLOOD CARBON DIOXIDE [1]

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The level of carbon dioxide in the blood is now generally accepted as one of the major factors in the regulation of the cerebral circulation. It was first shown in experimental animals that increase in blood carbon dioxide exerts a vasodilator effect on cerebral blood vessels (1, 2). Similar effects were postulated in man by Lennox and Gibbs (3) on the basis of decrease in the cerebral arteriovenous oxygen difference. Conclusive evidence of the effects of carbon dioxide on cerebral blood flow in man was provided by the experiments of Kety and Schmidt with the nitrous oxide method. Reduction in arterial CO_2 through hyperventilation was shown to produce a decrease in blood flow, (4) whereas increase in arterial CO_2 through inhalation of 5 per cent and 7 per cent carbon dioxide was found to cause a striking increase in blood flow (5).

The above observations on man, which were concerned with large-scale effects, do not in themselves permit a precise formulation of the role of carbon dioxide in the control of the cerebral circulation. The minimal changes in blood CO_2 which will evoke vascular responses must be known, together with the degrees of response which are produced by given increments of change in CO_2 beyond these threshold values. Knowledge of the minimal increase in arterial CO_2 required to dilate cerebral vessels has potential therapeutic application in the treatment of certain states of severe impairment of blood flow to the brain. Carbon dioxide in 5 per cent or greater concentration has the disadvantages of raising blood pressure (5, 6) and producing uncomfortable dyspnea within a relatively few minutes (7). It appeared possible, however, that some lower concentration of carbon dioxide might prove more tolerable, while still retaining vasodilator properties.

The present studies were concerned with the threshold of response of the cerebral vessels of man to increase in blood carbon dioxide. Cerebral blood flow determinations were made with the nitrous oxide method, employing concentrations of 2.5 and 3.5 per cent CO_2 in the inspired gas, and the associated changes in blood gases and pH were measured.

METHODS

The subjects for these studies were hospital patients convalescing from a variety of illnesses in which the brain was not involved. Seven patients and five normal volunteer subjects were given 2.5 per cent carbon dioxide by inhalation and 12 patients and seven normal subjects were given 3.5 per cent carbon dioxide. The mean ages of these two groups were 30 and 34 years, respectively. Cerebral blood flow (CBF) before and during carbon dioxide inhalations was determined by the nitrous oxide method (8) with slight modifications (9). Six of the 28 subjects were studied by measurements of cerebral arteriovenous gas differences alone and one subject (H. J.) separately by both techniques. Cerebral oxygen consumption (CMR_{O_2}) was determined from the cerebral blood flow multiplied by the cerebral arteriovenous oxygen difference, $(A\text{–}V)_{O_2}$. The values for $(A\text{–}V)_{O_2}$ were obtained from analyses of arterial and internal jugular blood samples drawn just before and just after the cerebral blood flow procedure and pooled. The cerebral vascular resistance (CVR) was calculated by dividing the blood flow into the mean arterial pressure, measured several times during the CBF procedure from either the femoral or brachial arteries with a damped mercury manometer.

Control observations of the cerebral circulation were made with the standard gas mixture for the nitrous oxide method (15 per cent N_2O, 21 per cent O_2, 64 per cent N_2). Following this the patient was given a mixture contain-

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TABLE I

Cerebral functions and blood studies during air and carbon dioxide breathing *

	Pt.	Age	CBF cc./100 Gm./min. Air	CBF cc./100 Gm./min. CO_2	$CMRO_2$ cc./100 Gm./min. Air	$CMRO_2$ cc./100 Gm./min. CO_2	CVR mm. Hg/cc./100 Gm./min. Air	CVR mm. Hg/cc./100 Gm./min. CO_2	Mean art. pressure mm. Hg Air	Mean art. pressure mm. Hg CO_2	$(A\text{-}V)O_2$ Vol. % Air	$(A\text{-}V)O_2$ Vol. % CO_2	Hematocrit	Art. pH Air	Art. pH CO_2	Art. pCO_2 mm. Hg Air	Art. pCO_2 mm. Hg CO_2	Venous pH mm. Hg Air	Venous pH mm. Hg CO_2	Venous pCO_2 mm. Hg Air	Venous pCO_2 mm. Hg CO_2	Venous pO_2 mm. Hg Air	Venous pO_2 mm. Hg CO_2
	W. M.	21	50	49	2.9	2.9	2.1	2.1	107	105	5.7	6.0	45										
	E. H.	29	45	61	2.1	3.1	1.6	1.2	73	74	3.7	5.1	38										
	M. B.	53	51	53	2.3	2.5	1.2	1.7	61	87	4.6	4.8	23										
	C. D.	19	56	52	2.8	3.1	1.4	1.6	80	84	5.0	6.0	45										
	V. H.	42	48	52	2.5	2.6	1.4	1.5	67	78	5.2	5.0	36										
2.5% CO_2	W. H.	19	62	57	3.9	3.4	1.4	1.5	85	86	6.4	5.9	45										
	T. R.	44	47	47	3.5	3.4	2.2	2.0	101	95	7.3	7.2	40										
	E. E.	37	43	48	2.6	3.1	2.4	2.0	104	96	6.1	6.5	40										
	B. G.	25	45	47	2.6	2.7	2.0	2.1	90	94	5.8	5.9	46	7.34	7.38	39	42	7.41	7.36	47	51	33	37
	H. W.	25	55	52	3.4	3.2	1.6	1.8	91	94	6.2	6.2	41	7.50	7.46	31	34	7.47	7.42	38	41	31	33
	J. D.	27	56	48	4.5	3.6	1.4	1.8	78	85	8.0	7.6	45	7.42	7.37	41	45	7.40	7.35	48	51	30	31
	W. N.	22	51	53	3.5	3.6	2.0	1.8	102	96	6.8	6.9	43	7.42	7.35	43	49	7.34	7.31	56	60	33	35
	Mean	30	51	52	3.1	3.1	1.7	1.8	87	90	5.9	6.1	41	7.44	7.39	38.4	42.5	7.41	7.36	47.2	50.7	31.7	34.0
	H. T.	23	49	63	3.1	2.5	1.5	1.3	72	78	6.2	3.9	37										
	C. C.	33	49	53	3.0	2.5	1.9	1.6	91	85	6.2	4.7	40	7.39	7.32	35	45	7.32	7.26	47	57	29	37
	R. L. D.	41	68	74	2.6	3.4	1.2	1.1	81	83	3.8	4.6	27										
	E. J.	46	58	53	3.2	2.7	1.9	2.1	108	109	5.6	5.1	33										
3.5% CO_2	W. McL.	25	51	54	3.3	3.5	1.9	1.5	94	83	6.5	6.4	47	7.38	7.30	47	55	7.30	7.25	61	70	33	35
	G. G.	40	68	72	3.3	3.4	2.1	1.8	144	126	4.8	4.7	40	7.40	7.36	43	47	7.34	7.32	52	55	34	37
	W. M. J.	36	57	54	3.0	2.9	1.6	1.8	90	96	5.1	5.3	38	7.42	7.40	37	39	7.37	7.36	46	47	35	35
	G. M.	52	30	33	2.6	2.7	3.6	3.4	110	112	8.6	8.3	44	7.40	7.35	41	45	7.33	7.29	52	54	26	27
	H. J.	33	49	64	2.6	3.1	1.8	1.4	86	87	5.4	4.9	41	7.39	7.35	37	42	7.38	7.33	43	48	32	38
	H. W.	25	42	58	3.1	2.9	2.5	1.7	106	99	7.5	5.1	39	7.43	7.38	33	41	7.41	7.40	43	48	28	37
	D. C.	32	50	54	3.3	3.1	1.8	1.6	91	88	6.7	6.2	40	7.36	7.35	43	44	7.37	7.33	47	51	31	35
	Mean	35	52	57†	3.0	3.0	2.0	1.8†	98	95	6.0	5.4†	39	7.40	7.35	39.4	44.7	7.35	3.32	48.8	53.8	31.0	35.1

* Individual pCO_2 values are rounded to nearest whole number. Figures for mean pCO_2 obtained from unrounded individual values (see Methods).
† Statistical significance of difference from mean with air: $p \gtrsim .05$ (calculated by analysis of variance with subject excluded from variable).

ing 2.5 per cent or 3.5 per cent CO_2, with 21 per cent O_2 and the remainder N_2. At the end of 15 to 20 minutes this mixture was changed to gas containing the same percentage of CO_2 and O_2, together with 15 per cent N_2O, and the experimental blood flow determination carried out.

The pooled blood samples of arterial and venous blood were analyzed for oxygen and carbon dioxide content by the combined procedure for both gases described by Peters and Van Slyke (10), as modified for the presence of nitrous oxide by Kety and Schmidt (8). Oxygen capacity of the blood samples was determined by the method of Roughton, Darling, and Root (11). Blood pH was measured with a Cambridge Model R. pH meter, with appropriate corrections to body temperature (12). Carbon dioxide tensions (pCO_2) were obtained from the pH, CO_2 content and hematocrit by means of the nomogram of Singer and Hastings (13). The individual pCO_2 values were estimated to the nearest 0.1 mm. Hg from an enlarged print of this nomogram. This was done to define as precisely as possible the blood flow—pCO_2 relations over a narrow range of CO_2 increase. No implication is intended, however, that the method possesses such accuracy in terms of the absolute magnitude of individual determinations. Venous oxygen tension was determined from the pH and the per cent of oxygen saturation, using the oxygen-hemoglobin dissociation curves of Dill (14). Observations were made on the character of the subject's breathing, but respiratory minute volumes were not measured.

RESULTS

Inhalation of 2.5 per cent carbon dioxide produced very little change in the mean values of the cerebral blood flow, cerebral oxygen consumption or vascular resistance (Table I). The mean arterial pH and pCO_2 values with air and CO_2 breathing were respectively: 7.44 and 7.39, 38.4 and 42.5 mm. Hg. A comparable group of seven subjects given 2.5 per cent CO_2 for 15 minutes showed a decrease in the mean value for arterial pH from 7.37 to 7.34 and an increase in arterial pCO_2 from 41.3 to 45.6 mm. Hg. Dyspnea and increase in rate or depth of the subjects' breathing were either slight or not detectable. The same concentration of carbon dioxide was also well tolerated by 10 patients with cerebral vascular accidents (7) for periods of 30 minutes to one hour.

Inhalation of carbon dioxide in 3.5 per cent concentration was associated with a mean increase of 10 per cent ($p < .05$) in cerebral blood flow and a comparable decrease in cerebral vascular resistance ($p < .05$). There was no change in cerebral oxygen consumption. There was, however, a decrease in the cerebral arteriovenous oxygen difference. The mean values of $(A–V)_{O_2}$ for air and CO_2 breathing in the patients studied by the nitrous oxide method were 6.0 and 5.4 volumes per cent ($p = .05$), respectively. In the larger group comprising 16 observations on 15 subjects, the mean value for $(A–V)_{O_2}$ was 6.4 volumes per cent with air and 5.5 volumes per cent with CO_2 breathing ($p < .01$). In 12 of these observations there was a fall in $(A–V)_{O_2}$ of 0.5 volumes per cent or more during CO_2 inhalation, and in four there was little change (Table II). Changes in the blood gas tensions and pH in the eight patients of the nitrous oxide group in whom these functions were studied were almost identical with those in the larger group of 15 subjects. In these 15 individuals 3.5 per cent carbon dioxide produced the following increases: arterial pCO_2, 5.3 mm. Hg; jugular venous pCO_2, 4.3 mm. Hg; jugular venous pO_2, 4.7 mm. Hg. Arterial pH fell .04 unit and jugular venous pH .03 unit with this concentration of carbon dioxide.

The relation between increase in arterial CO_2 tension and the corresponding change in two different measures of cerebral blood flow is shown in Figure 1. These measures are the cerebral blood flow as determined by the nitrous oxide method, and the cerebral blood flow as indicated by the reciprocal of the arteriovenous oxygen difference. In order to make them comparable, the values for both determinations during carbon dioxide inhalation are shown as percentage of control. Each of the points as plotted represents the mean obtained with a given concentration of inspired CO_2 in those subjects on whom both the CBF_{N_2O} and $\frac{1}{(A\text{-}V)_{O_2}}$ measurements were made. The function $\frac{1}{(A\text{-}V)_{O_2}}$ represents a valid measure of changes in blood flow where O_2 consumption is constant. The data for 5 per cent and 7 per cent CO_2 are taken from the work of Kety and Schmidt (5). However, supplementary observations made with 5 per cent CO_2 in our laboratory (7) in four patients with cerebral vascular accidents showed a decrease in the cerebral arteriovenous oxygen difference only slightly less than that observed by Kety and Schmidt in normal subjects.

It is evident in Figure 1 that a rather abrupt change in the slope of both curves must occur between the 2.5 per cent and 3.5 per cent inspired

TABLE II

Arterial and internal jugular blood gas and pH determinations in sixteen observations on fifteen subjects during air and 3.5 per cent carbon dioxide inhalation *

Patient	Age	Art. pCO_2 mm. Hg		Art. pH		Ven. pCO_2 mm. Hg		Ven. pH		Ven. pO_2 mm. Hg		$(A\text{-}V)_{O_2}$ Vol. %		$\frac{1}{(A\text{-}V)_{O_2}}$ as % of control (air)
		Air	CO_2	Air	CO_2	Air	CO_2	Air	CO_2	Air	CO_2	Air	CO_2	CO_2
C. C.	33	35	45	7.39	7.32	47	57	7.32	7.26	29	37	6.2	4.7	132
W. McL.	25	47	55	7.38	7.30	61	70	7.30	7.25	33	35	6.5	6.4	102
G. G.	40	43	47	7.40	7.36	52	55	7.34	7.32	34	37	4.8	4.7	102
W. M. J.	36	37	39	7.42	7.40	46	47	7.37	7.36	35	35	5.1	5.3	96
G. M.	52	41	45	7.40	7.35	52	54	7.33	7.29	26	27	8.6	8.3	104
C. P.	22	40	40	7.38	7.38	48	49	7.32	7.32	27	32	4.9	3.8	129
J. S.	25	42	49	7.39	7.38	51	55	7.35	7.34	35	38	5.3	4.8	110
C. H.	58	48	55	7.31	7.27	58	61	7.28	7.24	30	41	5.4	3.9	138
E. C.	37	37	45	7.43	7.37	52	57	7.35	7.32	24	28	9.6	7.8	123
H. J.	33	37	42	7.39	7.35	43	48	7.38	7.33	32	38	5.4	4.9	110
H. W.	25	32	41	7.43	7.38	42	48	7.41	7.36	28	37	7.5	5.1	147
D. C.	32	43	44	7.36	7.35	47	51	7.37	7.33	31	35	6.7	6.2	108
H. J.	33	38	44	7.40	7.35	46	50	7.37	7.33	38	46	4.6	3.6	126
B. G.	27	40	47	7.41	7.35	48	53	7.38	7.34	30	34	7.2	6.2	116
C. W.	26	41	42	7.39	7.36	51	53	7.35	7.32	32	35	7.7	7.2	108
G. H.	31	39	44	7.42	7.37	47	51	7.38	7.35	31	34	7.4	5.7	129
Mean	33	40.0	45.3†	7.39	7.35	49.4	53.7	7.35	7.32	30.9	35.6	6.4	5.5†	117.5

* Individual pCO_2 values are rounded to nearest whole number. Figures for mean pCO_2 obtained from unrounded individual values (see Methods).

† Tested by analysis of variance and found significantly different ($p < .01$) from mean value with air breathing.

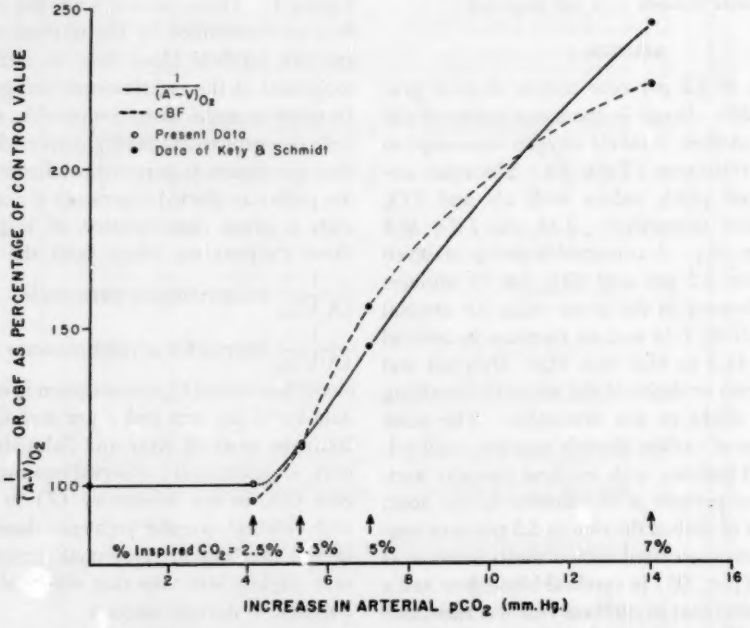

FIG. 1. RELATION BETWEEN THE CEREBRAL BLOOD FLOW AND RECIPROCAL OF THE CEREBRAL ARTERIOVENOUS OXYGEN DIFFERENCE AND THE INCREASE IN ARTERIAL CARBON DIOXIDE TENSION

carbon dioxide concentrations, and likewise between the increases in arterial pCO_2 produced by these concentrations. The progressive rise in both measures of cerebral blood flow with further increments in the inspired CO_2 concentration and the arterial pCO_2 is also readily apparent. A 12 to 13 per cent increase in blood flow in these subjects is indicated for a 5.3 mm. rise in pCO_2, and a doubling of blood flow is predicted for a 10 to 11 mm. Hg increase in CO_2 tension. In the larger group of 15 subjects, 3.5 per cent CO_2 produced a mean increase in $\frac{1}{(A\text{-}V)_{O_2}}$ of 17 per cent. The mean changes in arterial pCO_2 shown in this figure for the 2.5, 3.5, 5, and 7 per cent CO_2 concentrations are respectively: 4.1, 5.3, 7.0, and 14 mm. Hg. A scatter plot of the individual data shows no tendency for the value of $\frac{1}{(A\text{-}V)_{O_2}}$ to rise with increases in arterial pCO_2 smaller than 5 mm. Hg, but a clearly evident tendency for this function to rise with increases of pCO_2 of 5 mm. or greater. The same can be said for the individual CBF_{N_2O} data, although there are fewer points to indicate the trend of the relationship.

During the inhalation of 3.5 per cent carbon dioxide a definite deepening and slight increase in rate of respiration were usually observed within 10 to 15 minutes. These were associated with slight dyspnea, which in a few subjects became definitely uncomfortable within 30 minutes.

DISCUSSION

The threshold for cerebral vasodilator effect evidently lies beyond the 2.5 per cent inspired CO_2 concentration and its associated mean increase in arterial CO_2 tension of 4.1 mm. Hg. This conclusion is based on the absence of changes in the cerebral blood flow, vascular resistance and arteriovenous oxygen difference.

In regard to 3.5 per cent CO_2, the validity of the cerebral arteriovenous oxygen difference as a measure of small changes in blood flow, under the conditions of these experiments, should first be considered. Actually, the validity of change in $\frac{1}{(A\text{-}V)_{O_2}}$ as a measure of change in CBF rests upon the demonstration that cerebral oxygen consumption, as determined by the nitrous oxide method, is not altered by inhalation of carbon dioxide over the concentration range studied. Since $CBF = \frac{CMR_{O_2}}{(A\text{-}V)_{O_2}}$, it is evident that when CMR_{O_2} is a constant the value of $\frac{1}{(A\text{-}V)_{O_2}}$ will vary as the blood flow. There seems little reason to doubt the constancy of the CMR_{O_2}, since the number of subjects in the present series combined with those studied by Kety and Schmidt is relatively large. Random errors in the nitrous oxide method would tend to be averaged out, and even systematic errors would not preclude correct conclusions regarding the constancy of cerebral metabolism. It is quite possible that in a limited series of observations, the change in $\frac{1}{(A\text{-}V)_{O_2}}$ might be the more accurate measure of small changes in blood flow. The nitrous oxide method in any given determination contains more possibilities of error, since it involves multiple analyses, the assumption of equilibrium in respect to nitrous oxide between the brain and jugular venous blood, and other potential sources of error. In the present studies, these two measures of blood flow were for the most part in good agreement, although the larger number of oxygen difference determinations gives this measure somewhat greater statistical validity.

The relations shown in Figure 1 suggest that the cerebrovascular response to increase in arterial pCO_2 is a threshold type of phenomenon. The rise from the control level of both the CBF_{N_2O} and $\frac{1}{(A\text{-}V)_{O_2}}$ evidently occurs over a narrow range of increase in arterial CO_2 tension. Since mean arterial blood pressure was unaffected by 3.5 per cent CO_2 (Table I) and only slightly to moderately affected by 5 per cent and 7 per cent CO_2, (5) the rising curve of cerebral blood flow with increase in arterial CO_2 tension beyond threshold must have been due primarily to progressive dilatation of cerebral blood vessels.

The mean threshold value for increase in arterial pCO_2, below which there was no effect on cerebral vessels and above which there occurred progressive dilation, was approximately 4.5 mm. Hg. It can be seen that both of the curves of Figure 1 when extrapolated downward intersect the control (100 per cent) level very near this point. In the individual data no pCO_2 values lie between

4.2 and 5.0 mm. Hg, but in each of four subjects who had individual increases of 5.0 mm. Hg in pCO_2 the value of $\frac{1}{(A\text{-}V)_{O_2}}$ was considerably elevated above control level. This suggests that the 5.0 mm. Hg increase was beyond threshold. It should be emphasized that the 4.5 mm. Hg increase in pCO_2 represents the average threshold in the subject group studied, and that some individual and group variation would be expected.

It is apparent that inhalation of 3.5 per cent CO_2, with its associated mean increase in arterial pCO_2 of 5.3 mm. Hg, produced significant cerebral vasodilatation, since the CBF_{N_2O}, $\frac{1}{(A\text{-}V)_{O_2}}$ and the calculated CVR all showed statistically significant changes. Only four of 15 subjects, including the only patient with definite hypertension (C. G.) showed an increase in $\frac{1}{(A\text{-}V)_{O_2}}$ smaller than eight per cent. The difference between the pCo_2 change with 3.5 per cent CO_2 and that produced by 2.5 per cent appears surprisingly small in relation to the difference in effect. However, the importance of small changes in pCO_2 beyond threshold is emphasized by the increase in blood flow of approximately 50 per cent which is produced by a 7 mm. Hg increase in CO_2 tension (8).

Although the threshold defined above has been stated in terms of change in arterial pCO_2, it is possible that it actually represents a threshold for the effects of associated change in hydrogen ion or bicarbonate ion concentration. The work of Schieve and Wilson (15) makes it appear unlikely that the H-ion has any cerebral vasodilator effect, but does not exclude the HCO_3-ion. The question must also be raised whether we are dealing with a "pure" CO_2 threshold or whether an increase in oxygen tension as a result of hyperventilation might have exerted opposing (vasoconstrictor) effect. However, cerebral vessels are only slightly constricted by 50 per cent oxygen (16) which, on the basis of the alveolar equation (17) and an assumed alveolar-arterial pO_2 gradient of 20 mm. Hg, should produce an arterial pO_2 of nearly 300 mm. Hg. Since the inspired pO_2 itself in these experiments was not over 159 mm. Hg, the possibility appears remote that change in arterial pO_2 influenced the results.

The site of action of the carbon dioxide in these experiments was probably the blood vessels themselves. Although an autonomic vasodilator innervation has been demonstrated in the experimental animal (18), its functional significance for man is unknown. It is of interest that recent experiments suggest that a threshold concentration of CO_2 is required for respiratory stimulation (19, 20). Observations have been made on the effect of an increase in blood CO_2 on other vessels of the body deprived of their vasomotor innervation. The vessels in sympathectomized upper extremities in man (21) and the hind limb vessels in dogs, given ganglionic-blocking doses of tetraethylammonium (22), respond alike by vasodilatation to increase in blood carbon dioxide.

The CO_2 tension threshold for cerebral vascular effect which is reported in this paper is nonspecific in the sense that it does not indicate which type of vessel was responding: arteries, arterioles, capillaries or venules. Since the increases in venous pCO_2 during 2.5 and 3.5 per cent CO_2 breathing were only slightly smaller than those in arterial pCO_2, the threshold as given would not be greatly in error, regardless of the type of vessel involved. As a corollary we may conclude that, if all of these vessels were responding, the threshold was nearly the same throughout the group. In the case of diseased cerebral blood vessels, or of vessels adapted to chronic abnormality in blood pCO_2, it might be anticipated that the thresholds of response to CO_2 change would show some differences from those of normal vessels. In cerebral arteriosclerosis the vascular response to 5 per cent CO_2 inhalation has been shown to be diminished (23).

In the intrinsic control of the cerebral circulation, it appears probable that carbon dioxide and oxygen tension changes operate simultaneously, and usually in the same direction rather than in competition. Thus, with a fall in cerebral blood flow, the resulting fall in capillary and venous pO_2 and rise in pCO_2 both represent vasodilator stimuli. The threshold for the combined vascular effects of fall in pO_2 and rise in pCO_2 quite likely is reached at a smaller value for pCO_2 increase than the threshold reported in this paper. From the Fick equation, and the blood nomogram (13) or the physiological CO_2 absorption curve (24), it can be shown that, with CMR_{O_2} constant, cerebral

blood flow must fall by approximately 30 per cent to raise venous pCO_2 to the vasodilator threshold. There is some evidence that vessels actually dilate with a smaller reduction in blood flow (25). Studies on such combined thresholds, and on the threshold of cerebral vascular response to reduction in blood CO_2 tension, obviously are needed. The data in the existing literature on the combined effects of arterial pCO_2 and pO_2 on cerebral blood flow have recently been worked into a useful nomogram by Cannon (26).

Therapeutic applications of our findings remain to be explored. Carbon dioxide in 3.5 per cent concentration appears potentially useful for the treatment of cerebral vascular manifestations produced by inadequate blood flow. Although more weakly vasodilator than 5 per cent CO_2, it is considerably more tolerable from the standpoint of dyspnea and can be given for 30 minutes to most patients without producing excessive dyspnea or increases in arterial blood pressures. Its trial in selected patients with cerebral vascular insufficiency seems indicated.

SUMMARY

1. The vasodilator response of normal cerebral vessels to rapid increase in arterial carbon dioxide tension appears to be a threshold type of phenomenon.

2. In a group of 28 subjects, a mean increase in arterial CO_2 tension of less than 4.5 mm. Hg was without vascular effect, whereas increases greater than this value produced progressive vasodilatation.

3. With constant cerebral metabolism, a reduction in blood flow of approximately 30 per cent would be required to raise end-capillary and venous pCO_2 to the vasodilator threshold for this gas.

4. Inhalation of 3.5 per cent carbon dioxide produced a 10 per cent mean increase in cerebral blood flow, but little change in blood pressure in most subjects. It causes considerably less dyspnea than 5 per cent CO_2, and may have application in the treatment of states of cerebral vascular insufficiency.

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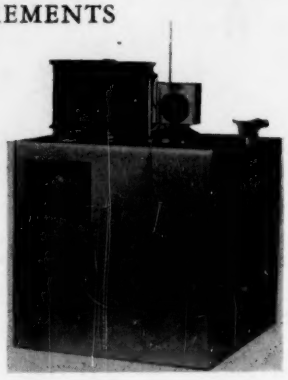

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